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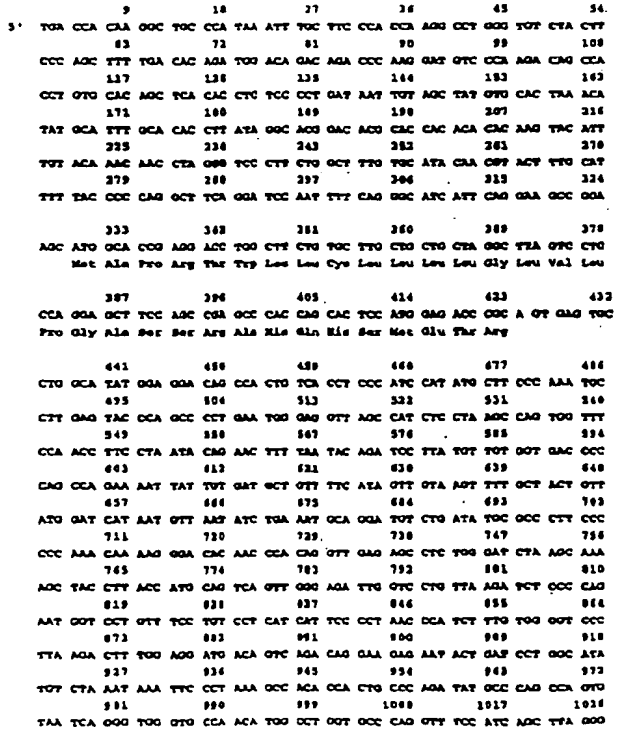
(54) Title: LIGAND POLYPEPTIDE FOR THE G PROTEIN-COUPLED RECEPTOR FROM HUMAN PITUITARY GLANDS

(57) Abstract

The present invention relates to the murine-derived ligand polypeptide for the G protein-coupled receptor proteins. The ligand polypeptide or the DNA which codes for the ligand polypeptide can be used for (1) development of medicines such as pituitary function modulators, central nervous system function modulators, and pancreatic function modulators, (2) development of receptor binding assay systems using the expression of recombinant receptor proteins and screening of pharmaceutical candidate compounds, and (3) production of non-human transgenic animals or non-human knockout animals for analyzing a function of the genes.



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DESCRIPTION

LIGAND POLYPEPTIDE FOR THE G PROTEIN-COUPLED RECEPTOR FROM HUMAN PITUITARY GLANDS

[Technical Field]

The present invention relates to a novel ligand polypeptide for the G protein-coupled receptor protein and a DNA comprising a DNA encoding the ligand polypeptide.

[Background Art]

Many hormones and neurotransmitters mediate biological functions through specific receptors present on the cell membrane. Many of these receptors engage themselves in the intracellular transduction of signals through activation of the coupled guanine nucleotide-binding protein (hereinafter sometimes referred to briefly as G protein) and have the common structure comprising 7 transmembrane domains. Therefore, these receptors are collectively referred to as G protein-coupled receptor or 7-transmembrane receptor.

One of the pathways to modulate biological functions mediated by such hormones or neurotransmitters through G protein-coupled receptors is the hypothalamo-pituitary system. Thus, the secretion of pituitary hormones from the hypophysis is controlled by hypothalamic hormones (pituitatropic releasing factor) and the functions of the target cells or organs are regulated through the pituitary hormones released into the circulation. This pathway carries out functional modulations of importance to the living body, such as homeostasis and regulation of the reproduction, development, metabolism and growth of individuals. The secretion of pituitary hormones is controlled by a positive feedback or a negative feedback mechanism involving hypothalamic hormones and the peripheral hormone secreted from the target endocrine gland. The

various receptor proteins present in the hypophysis are playing a central role in the regulation of the hypothalamus-pituitary system.

Meanwhile, it is known that these hormones and factors as well as their receptors are not localized in the hypothalamus-pituitary system but are broadly distributed in the brain. Therefore, it is suspected that, in the central nervous system, this substance called hypothalamus hormone is functioning as a neurotransmitter or a neuromodulator. Moreover, the substance is distributed in peripheral tissues as well and thought to be playing important roles in the respective tissue.

The pancreas is playing a crucial role in the carbohydrate metabolism by secreting glucagon and insulin as well as digestive juice. While insulin is secreted from the pancreatic β cells, its secretion is mainly stimulated by glucose. However, it is known that β cells have a variety of receptors and the secretion of insulin is controlled by a number of factors in addition to glucose as well as peptide hormones, e.g. galanine, somatostatin, gastric inhibitory polypeptide, glucagon, amyrin, etc.; sugars, e.g. mannose etc.; amino acids, and neurotransmitters, among others.

The means only heretofore available for identifying ligands for said G protein-coupled receptor proteins is estimation from the homology in primary structure of G protein-coupled receptor proteins.

Recently, investigation for novel opioid peptides by introducing a cDNA coding for a receptor protein which a ligand is unknown, i.e. an orphan G protein-coupled receptor protein, into CHO cells have been reported (Reinsheid, R. K. et al., Science, 270, 792-794, 1995, Menular, J.-C., et al., Nature 377, 532-535, 1995). However, in view of similarities to known G

protein-coupled receptor proteins and tissue distributions, it could be easily anticipated in these cases that the ligand would be belonging to the family of opioid peptides. The history of research and development in the realm of substances acting on the living body through the opioid receptor dates back to many years ago and various antagonists and agonists had been developed. Therefore, among the compounds artificially synthesized, an agonist of the receptor was picked out and, using it as a probe, expression of the receptor in the receptor cDNA-transfected cells was verified. Then, a search was made for an activator of the intracellular signal transduction which was similar to the agonist, and the activator so found was purified, and then the structure of the ligand was determined. However, when the homology of an orphan receptor to known G protein-coupled receptor proteins is low, little information is available on the ligand so that it is difficult to explore the ligand.

Belonging to the category of such orphan (i.e. of unknown ligand) G protein-coupled receptor proteins, a human receptor protein encoded by phGR3 (alias GPR10) gene [Genomics, 29, 335, 1995] and the corresponding rat receptor protein UHR-1 [Biochem. Biophys. Res. comun., 209, 606, 1995] are known.

[Disclosure of Invension]

As an approach toward elucidation of the mechanisms of onset of various diseases and establishment of pertinent therapeutics, it is relevant and rewarding to determine the ligands of orphan G protein-coupled receptor proteins which are considered to be show some physiological functions or others in the living body. For example, ligands binding the orphan G protein-coupled receptor proteins expressed in the pituitary, central nervous system, and pancreatic β

cells, among others, are expected to be useful drugs, although their structures and functions remain to be fully known.

Meanwhile, in order to analyze the function of a ligand, it is a very effective procedure to compare the case in which the relevant ligand gene has been expressed in excess or the case in which the gene product ligand is functionally defective with the case in which the gene expression is normal. In this connection, the most reliable method for creating a functional defect in a given gene product is to destroy the very gene. For this purpose, the gene targeting technology was developed (Thomas, K. R. et al., Cell, 51, 503-512, 1987) and many knockout mice have been constructed in the world. This technology comprises destroying a given chromosomal gene of the pluripotent murine embryonic stem cell (ES cell) by way of a homologous recombination, microinjecting the resulting ES cell into the murine blastocyst to construct chimera mice, and mating them to produce a knockout mouse. At the present time only mice are available as such knockout animal models of various diseases. In order to construct a useful transgenic mouse with a defect in the gene which appears to be associated with a given disease, the mouse genomic DNA sequence of the target gene must be known but, for the orphan G protein-coupled receptor protein pHGR3 (GPR10) or UHR-1, neither the corresponding mouse ligand peptide nor the gene (cDNA or genomic DNA) coding for the ligand peptide is known.

By using cells in which the cDNA coding for the orphan G protein-coupled receptor protein pHGR3 has been expressed and selecting a specific cell stimulating (signal transduction) activity as an indicator, the inventors of the present invention performed a screening for a bovine polypeptide which

this receptor protein recognizes as its ligand and determined its amino acid sequence and DNA sequence. Then, using the DNA coding for said bovine polypeptide as a primer, the inventors isolated the bovine, human, and rat cDNAs coding for said polypeptide by polymerase chain reaction (PCR), synthesized primers based on the uncleotide sequence of the rat cDNA, and succeeded in isolating the cDNA and genomic DNA coding for the mouse polypeptide.

Furthermore, by using said ligand peptide, the inventors established a screening method for a compound which modifies the binding of the ligand to said receptor protein and further by using the genomic DNA of said mouse ligand polypeptide, constructed a non-human transgenic animal (particularly a useful knockout mouse) to thereby may it possible to analyze the function of the gene.

The present invention, therefore, relates to

(1) a polypeptide comprising an amino acid sequence represented by SEQ ID NO:1, or a substantial equivalent thereto, or its amide or ester, or a salt thereof,

(2) a DNA comprising a DNA having a nucleotide sequence coding for the polypeptide according to the above item (1),

(3) a DNA according to the above item (2), which comprises a nucleotide sequence represented by SEQ ID NO:2 or SEQ ID NO:3,

(4) a recombinant vector comprising the DNA according to the above item (2),

(5) a transformant which is transformed by the DNA according to the above item (2) or the recombinant vector according to the above item (4),

(6) a non-human knock out animal having an inactivated DNA of the DNA according to the above item (2),

(7) a non-human transgenic animal having the DNA according to the above item (2) or its mutein, or the

recombinant vector according to the above item (4),
(8) a non-human animal cell having an inactivated DNA of the DNA according to the above item (2),
(9) a method for producing the non-human animal cell according to the above item (8), which comprises introducing an inactivated DNA of the DNA according to the above item (2),
(10) a method for producing the polypeptide according to the above item (1) or its amide or ester, or the salt thereof, which comprises cultivating the transformant according to the above item (5) to produce and accumulate the polypeptide according to the above item (1), and collecting the same,
(11) a pharmaceutical composition which comprises the polipeptide according to the above item (1) or its amide or ester, or the salt thereof, and
(12) an antibody against the polypeptide according to the above item (1) or its amide or ester, or the salt thereof.

Further, this invention relates to an agent for treating or preventing dementia, depression (melancholia), hyperkinetic (microencephalo-pathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease, hyperphagia, polyphagia, hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, diabetes, cancer, pancreatitis, renal disease, Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility and/or oligogalactia.

In this specification, the term "substantial equivalent(s)" means that the activity of the protein, e.g., nature of the binding activity of the ligand and

the receptor and physical characteristics are substantially the same. Substitutions, deletions, additions or insertions of amino acids often do not produce radical changes in the physical and chemical characteristics of a polypeptide, in which case polypeptides containing the substitutions, deletions, additions or insertions would be considered to be substantially equivalent to polypeptides lacking the substitutions, deletions, additions or insertions. Substantially equivalent substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In this specification, a mutated polypeptide which is obtained by a mutation (such as substitution, deletion, addition or insertion etc.) of the non-mutated polypeptide is a polypeptide substantially the same as the non-mutated polypeptide, wherein the physiological characteristics and chemical characteristics of the non-mutated polypeptide is not effected by the mutation.

The polypeptide of the present invention represents a precursor polypeptide of a matured ligand polypeptide (e.g. a polypeptide comprising an amino acid sequence represented by SEQ ID NO:4 or a substantial equivalent thereof) which can bind to the G protein-coupled receptors.

For example, the polypeptide of the present invention represents a polypeptide which comprises an

amino acid sequence represented by SEQ ID NO:1 or its substantial equivalent thereto, or its amide or ester, or a salt thereof (hereinafter, sometimes referred to briefly as ligand polypeptide or polypeptide). However, the polypeptide consisting of an amino acid sequence represented by SEQ ID NO:4 is excluded from the polypeptide of the present invention.

[Brief Description of the Drawings]

Fig. 1 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino acid encoded by the nucleotide sequence. The primer used for sequencing was -21M13. The underscored region corresponds to the synthetic primer.

Fig. 2 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino acid sequence encoded thereby. The primer used for sequencing was M13RV-N (Takara). The underscored region corresponds to the synthetic primer.

Fig. 3 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 1.

Fig. 4 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 2.

Fig. 5 is a diagram comparing the partial amino acid sequence of the protein encoded by the human pituitary-derived G protein-coupled receptor protein

cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 with the known G protein-coupled receptor protein S12863. The shadowed region represents the region of agreement. The 1st to 9th amino acid sequence of p19P2 corresponds to the 1st to 99th amino acid sequence of Fig. 1 and the 156th to 230th amino acid sequence corresponds to the 1st to 68th amino acid sequence of Fig. 2.

Fig. 6 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment based on the nucleotide sequences of the MIN6-derived G protein-coupled receptor protein cDNA fragments harbored in the cDNA clones pG3-2 and pG1-10 isolated by PCR using MIN6-derived cDNA and the amino acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 7 is a diagram comparing the partial amino acid sequence encoded by pG3-2/pG1-10 of the MIN6-derived G protein-coupled receptor protein shown in Fig. 6 with the partial amino acid sequence of the protein encoded by p19P2 shown in Figs. 1 and 2. The shadowed region corresponds to the region of agreement. The 1st to 99th amino acid sequence of the protein encoded by p19P2 corresponds to the 1st to 99th amino acid sequence of Fig. 1 and the 156th to 223rd amino acid sequence corresponds to the 1st to 68th amino acid sequence of Fig. 2. The 1st to 223rd amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1st to 223rd amino acid sequence of Fig. 6.

Fig. 8 is a partial hydrophobic plot of the MIN6-derived G protein-coupled receptor protein constructed according to the partial amino acid sequence shown in Fig. 6.

Fig. 9 shows the entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor

protein cDNA harbored in the cDNA clone phGR3 isolated from a human pituitary-derived cDNA library by the plaque hybridization method using the DNA fragment inserted in p19P2 as a probe and the amino acid sequence encoded by the nucleotide sequence.

Fig. 10 shows the results of Northern blotting of human pituitary mRNA hybridized with radioisotope-labeled human pituitary cDNA clone phGR3.

Fig. 11 shows a hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA harbored in the phGR3 as constructed according to the amino acid sequence shown in Fig. 9.

Fig. 12 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment harbored in the cDNA clone p5S38 isolated by PCR using MIN6-derived cDNA and the amino acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 13 shows a diagram comparing the partial amino acid sequence of MIN6-derived G protein-coupled receptor protein encoded by p5S38 shown in Fig. 12 with the partial amino acid sequence of G protein-coupled receptor protein encoded by the cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 and the partial amino acid sequence of G protein-coupled receptor protein encoded by the nucleotide sequence generated from the nucleotide sequences of cDNA fragments contained in pG3-2 and pG1-10 shown in Fig. 6. The shadowed region represents the sequence region of agreement. The 1st to 144th amino acid sequence of the protein encoded by p5S38 corresponds to the 1st to 144th amino acid sequence of Fig. 12, the 1st to 99th amino acid sequence of the protein encoded by p19P2 corresponds to the 1st to 99th amino acid sequence of Fig. 1 and the 156th to 223rd amino acid sequence

corresponds to 1st to 68th amino acid sequence of Fig. 2. The 1st to 223rd amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1st to 223rd amino acid sequence of Fig. 6.

Fig. 14 shows a partial hydrophobic plot of the protein encoded by the MIN6-derived G protein-coupled receptor protein cDNA harbored in p5S38 as constructed according to the partial amino acid sequence shown in Fig. 12.

Fig. 15 shows the results of the following analysis. Thus, RT-PCR was carried out to confirm the expression of mRNA in CHO cells transfected by pAKKO-19P2. Lanes 1-7 represent the results obtained by performing PCRs using serial dilutions of pAKKO-19P2 for comparison, i.e. the 10μl/ml stock solution (lane 1), 1/2 dilution (lane 2), 1/4 dilution (lane 3), 1/64 dilution (Lane 4), 1/256 dilution (lane 5), 1/1024 dilution (lane 6), and 1/4096 dilution (lane 7) of the plasmid as templates, and analyzing the reaction mixtures by 1.2% agarose gel electrophoresis. Lanes 8 through 11 are the results obtained by performing PCRs using a 1/10 dilution (lane 8), a 1/100 dilution (lane 9), and a 1/1000 dilution (lane 10) of the cDNA prepared from the CHO-19P2 cell line as templates and subjecting the respective reaction mixtures to electrophoresis. Lane 11 was obtained by performing PCR using a template obtained by carrying out cDNA synthesis without reverse transcriptase and subjecting the PCR reaction product to electrophoresis. Lanes 12 and 13 were obtained by performing PCR using cDNAs prepared from mock CHO cells with and without addition of reverse transcriptase, respectively, as templates and subjecting the respective reaction products to electrophoresis. M represents the DNA size marker. The lanes at both ends were obtained by electrophoresing 1 μl of λ/Sty I digest (Nippon Gene)

and the second lane from right was obtained with 1 µl of ø/χ174/Hinc II digest (Nippon Gene). The arrowmark indicates the position of the band amplified by PCR of about 400 bp.

Fig. 16 shows the activity of the crude ligand peptide fraction extracted from rat whole brain to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite releasing activity was expressed as % of the amount of [^{3}H] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [^{3}H] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30% CH_3CN fraction.

Fig. 17 shows the activity of the crude ligand polypeptide fraction extracted from bovine hypothalamus to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite release-promoting activity was expressed as % of the amount of [^{3}H] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [^{3}H] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30% CH_3CN fraction just as in the crude ligand polypeptide fraction from rat whole brain.

Fig. 18 shows the activity of the fraction purified with the reversed-phase column C18 218TP5415 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The active fraction from RESOURCE S was fractionated on C18 218TP5415. Thus, chromatography was carried out at a flow rate of

1 ml/min. on a concentration gradient of 20%-30% CH_3CN/0.1% TFA/H_2O, the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then, the activity of each fraction to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was determined. As a result, the activity was fractionated into 3 fractions (designated, in the order of elution, as P-1, P-2, and P-3).

Fig. 19 shows the activity of the fraction purified with the diphenyl 219TP5415 reversed-phase column to specifically promote arachidonic acid metabolite release from CHO-19P2 cells. The P-3 active fraction from C18 218TP5415 was fractionated on diphenyl 219TP5415. The chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 22%-25% CH_3CN/0.1% TFA/H_2O, the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity converged in a single peak.

Fig. 20 shows the activity of the fraction purified by μRPC C2/C18 SC 2.1/10 reversed-phase column to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The peak active fraction from diphenyl 219TP5415 was fractionated on μRPC C2/C18 SC 2.1/10. The chromatography was carried out at a flow rate of 100 μl/min. on a concentration gradient of 22%-23.5% CH_3CN/0.1% TFA/H_2O, the eluate was collected in 100 μl fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as two peaks of apparently a single substance (peptide).

Fig. 21 shows the activity of the P-2 fraction

purified by μRPC C2/C18 SC 2.1/10 reversed-phase column to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The chromatography was carried out at a flow rate of 100 μl/min. on a concentration gradient of 21.5%-23.0% CH_3CN/0.1% TFA/dH_2O, the eluate was collected in 100 μl fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as a peak of apparently a single substance.

Fig. 22 shows the nucleotide sequence of bovine hypothalamus ligand polypeptide cDNA fragment as derived from the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 23 shows the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment generated according to the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 24 shows the amino acid sequences (a) and (b) of the bovine hypothalamus-derived ligand polypeptides which specifically promote release of arachidonic acid metabolites from CHO-19P2 cells and the cDNA sequence

coding for the full coding region of the ligand polypeptides defined by SEQ ID NO:1 and SEQ ID NO:44.

Fig. 25 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed dH_2O at a final concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-31 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a concentration-dependent manner.

Fig. 26 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31(O)) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic ligand peptide was dissolved in degassed dH_2O at a final concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L31(O) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 27 shows the activity of synthetic ligand polypeptide 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed distilled water at a final concentration of 10^{-3}M and diluted

with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 28 shows the 1.2% agarose gel electrophoregram of the DNA fragments of the phages cloned from a bovine genomic library as digested with restriction enzymes BamHI(B) and SalI(S). As the DNA size marker (M), StyI digests of λ phage DNA were used. In lane B, two bands derived from the vector were detected in positions between the first (19,329 bp) and second (7.743 bp) marker bands, as well as 3 bands derived from the inserted fragment between the third (6,223 bp) and 5th (3,472 bp) bands. In lane S, two bands derived from the vector were similarly detected but due to the overlap of the band of the inserted fragment, the upper band is thicker than the band in lane B.

Fig. 29 shows the nucleotide sequence around the coding region as decoded from bovine genomic DNA. The 1st to 3rd bases (ATG) correspond to the translation start codon and the 767th to 769th bases (TAA) correspond to the translation end codon.

Fig. 30 shows a comparison between the nucleotide sequence (genome) around the coding region as deduced from bovine genomic DNA and the nucleotide sequence (cDNA) of bovine cDNA cloned by PCR. The sequence region of agreement is indicated by shading. As to the 101st to 572nd region, there is no corresponding region in the nucleotide sequence of cDNA, indicating that it is an intron.

Fig. 31 shows the translation of the amino acid

sequence encoded after elimination of the intron from the nucleotide sequence around the coding region as decoded from bovine genomic DNA.

Fig. 32 shows the full-length amino acid sequence and the cDNA sequence coding for the full coding region of rat ligand polypeptide.

Fig. 33 shows the amino acid sequence of bovine ligand polypeptide and the nucleotide sequences of DNAs coding for bovine polypeptide and rat polypeptide. The arrowmark indicates the region corresponding to the synthetic primer.

Fig. 34 shows the full-length amino acid sequence and the sequence of cDNA coding for the full coding region of human ligand polypeptide.

Fig. 35 shows a comparison of the amino acid sequences in the translation region of bovine ligand polypeptide, rat ligand polypeptide, and human ligand polypeptide.

Fig. 36 shows the nucleotide sequence of the inserted fragment of plasmid pmGB3. The arrowmark → indicates the sequence corresponding to the primer.

Fig. 37 shows the cDNA predicted from nucleotide sequence of plasmid pmGB3 and the predicted translated protein. The arrowmark → indicates the sequence corresponding to the primer. The sequence between the marks ‖ is the sequence predicted to be the intron.

Fig. 38 shows (i) the nucleotide sequence coding the ligand polypeptide of the present invention and its non-coding region, and (ii) the amino acid sequence of the ligand polypeptide of the present invention, which obtained in Example 34.

Fig. 39 shows the restriction enzyme map of the ligand polypeptide of the present invention.

Fig. 40 shows the construction figure for the targeting vector pmGFEN28 obtained in Example 35.

Fig. 41 shows the result of the agarose gel

electrophoresis described in Example 36, and the comparative gene map between the wild type and the recombinant (knock out) type.

[Best Mode for Carrying Out the Invention]

The polypeptide, its amide or ester, or a salt thereof (hereinafter sometimes referred to briefly as the ligand polypeptide or the polypeptide), processes for their production, and uses for the polypeptide are now described in detail.

The above ligand polypeptide of the present invention includes any polypeptides derived from any tissues, e.g. pituitary gland, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow, adrenal gland, skin, muscle, lung, digestive canal, blood vessel, heart, etc.; or cells of man and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine; sheep, bovine, monkey, etc. and comprising an amino acid sequence represented by SEQ ID NO:1 or a substantial equivalent thereto. For example, in addition to the protein comprising the amino acid sequence of SEQ ID NO:1, the ligand polypeptide of the present invention includes the protein comprising an amino acid sequence having a homology of about 50-99.9%, preferably 70-99.9%, more preferably 80-99.9% and especially preferably 90-99.9% to the amino acid sequence of SEQ ID NO:1 and having qualitatively substantially equivalent activity to the protein comprising the amino acid sequence of SEQ ID NO:1. The term "substantially equivalent" means the nature of the receptor-binding activity, signal transduction activity and the like is equivalent. Thus, it is allowable that even differences among grades such as the strength of receptor binding activity and the molecular weight of the polypeptide are present.

To be more specific, the ligand polypeptide of the

present invention includes the polypeptide derived from mouse and comprising the amino acid sequence of SEQ ID NO:1. In addition, the ligand polypeptide of the present invention includes the polypeptides which comprises substantially equivalent polypeptides such as (i) polypeptides wherein 1 to 15, preferably 1 to 10, and more preferably 1 to 5 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:1, (ii) polypeptides wherein 1 to 80, preferably 1 to 50, more preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:1, or polypeptides wherein 1 to 15, preferably 1 to 10, more preferably 1 to 5 amino acid residues are substituted with other amino acid residues.

Moreover, the ligand polypeptide of the present invention includes the polypeptides wherein its constructive amino acid (especially its side chain) is modified, or its amide or ester, or a salt thereof.

The polypeptide of the present invention includes those wherein Gln of the constitutive amino acid at the N-terminal side is cleaved in vivo to form pyroglutamyl group.

The peptides described in this specification, the left end is the N-terminus (amino terminus) and the right end is the C-terminus (carboxyl terminus) according to the convention of the peptide indication. While the C-terminus of the polypeptide of SEQ ID NO:1 is usually carboxyl (-COOH) or carboxylato ($-COO^-$), it may be amide ($-CONH_2$) or ester (-COOR) form. The ester residue R includes a C_{1-6} alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc., a C_{3-8} cycloalkyl group such as cyclopentyl, cyclohexyl, etc., a C_{6-12} aryl group such as phenyl, α-naphthyl, etc., and a C_{7-14} aralkyl group such as a phenyl-C_{1-2} alkyl group, e.g. benzyl, phenethyl, benzhydryl, etc. or an α-naphthyl-C_{1-2} alkyl, e.g. α-naphthylmethyl etc. In

addition, the ester may be a pivaloyloxymethyl ester which is broadly used for oral administration. When the polypeptide of SEQ ID NO:1 has a carboxyl or carboxylato group in any position other than the C-terminus, the corresponding amide or ester are also included in the concept of the polypeptide of the present invention. The ester mentioned just above includes the esters mentioned for the C-terminus.

The salt of polypeptide of the present invention includes salts with physiologically acceptable bases, e.g. alkali metals or acids such as organic or inorganic acids, and is preferably a physiologically acceptable acid addition salt. Examples of such salts are salts thereof with inorganic acids, e.g. hydrochloric acid, phosphoric acid, hydrobromic acid or sulfuric acid, etc. and salts thereof with organic acids, e.g. acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid or benzenesulfonic acid, etc.

The ligand polypeptide or amide or ester, or a salt thereof of the present invention may be (i) manufactured from the tissues or cells of warm-blooded animals inclusive of human by purifying techniques or (ii) manufactured by the peptide synthesis as described hereinafter. Moreover, (iii) it can be manufactured by culturing a transformant carrying a DNA coding for the polypeptide as described hereinafter.

In the production from the tissues or cells of human or other warm-blooded animals, the ligand polypeptide can be purified and isolated by a process which comprises homogenizing the tissue or cells of human or other warm-blooded animal, extracting the homogenate with an acid, for instance, and subjecting the extract to a combination of chromatographic

procedures such as reversed-phase chromatography, ion-exchange chromatography, affinity chromatography, etc.

As mentioned above, the ligand polypeptide in the present invention can be produced by per se known procedures for peptide synthesis. The methods for peptide synthesis may be any of a solid-phase synthesis and a liquid-phase synthesis. Thus, the objective peptide can be produced by condensing a partial peptide or amino acid capable of constituting the protein with the residual part thereof and, when the product has a protective group, the protective group is removed whereupon a desired peptide can be manufactured. The known methods for condensation and deprotection includes the procedures described in the following literature (1)-(5).

(1) M. Bodanszky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York, 1966

(2) Schroeder and Luebke, The Peptide, Academic Press, New York, 1965

(3) Nobuo Izumiya et al., Fundamentals and Experiments in Peptide Synthesis, Maruzen, 1975

(4) Haruaki Yajima and Shumpei Sakakibara, Biochemical Experiment Series 1, Protein Chemistry IV, 205, 1977

(5) Haruaki Yajima (ed.), Development of Drugs-Continued, 14, Peptide Synthesis, Hirokawa Shoten

After the reaction, the protein can be purified and isolated by a combination of conventional purification techniques such as solvent extraction, column chromatography, liquid chromatography, and recrystallization. Where the protein isolated as above is in a free form, it can be converted to a suitable salt by the known method. Conversely where the isolated product is a salt, it can be converted to the free peptide by the known method.

The amide of polypeptide can be obtained by using a resin for peptide synthesis which is suited for amidation. The resin includes chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmethylphenylacetamidomethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin, and so on. Using such a resin, amino acids whose α-amino groups and functional groups of side-chain have been suitably protected are condensed on the resin according to the sequence of the objective peptide by various condensation techniques which are known per se. At the end of the series of reactions, the peptide or the protected peptide is sepasated from the resin and the protective groups are removed to obtain the objective polypeptide.

For the condensation of the above-mentioned protected amino acids, a variety of activating reagents for peptide synthesis can be used but a carbodiimide compound is particularly suitable. The carbodiimide includes DCC, N,N'-diisopropylcarbodiimide, and N-ethyl-N'-(3-dimethylaminoprolyl)carbodiimide. For activation with such a reagent, a racemization inhibitor additive, e.g. HOBt and the protected amino acid are directly added to the resin or the protected amino acid pre-activated as symmetric acid anhydride, HOBt ester, or HOOBt ester is added to the resin. The solvent for the activation of protected amino acids or condensation with the resin can be properly selected from among those solvents which are known to be useful for peptide condensation reactions. For example, N,N-dimethylformamide, N-methylpyrrolidone, chloroform, trifluoroethanol, dimethyl sulfoxide, DMF, pyridine, dioxane, methylene chloride, tetrahydrofuran,

acetonitrile, ethyl acetate, or suitable mixtures of them can be mentioned. The reaction temperature can be selected from the range hitherto-known to be useful for peptide bond formation and is usually selected from the range of about -20°C - 50°C. The activated amino acid derivative is generally used in a proportion of 1.5-4 fold excess. If the condensation is found to be insufficient by a test utilizing the ninhydrin reaction, the condensation reaction can be repeated to achieve a sufficient condensation without removing the protective group. If repeated condensation still fails to provide a sufficient degree of condensation, the unreacted amino group can be acetylated with acetic anhydride or acetylimidazole.

The protecting group of amino group for the starting material amino acid includes Z, Boc, tertiary-amyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulfenyl, diphenylphosphinothioyl, or Fmoc. The carboxy-protecting group that can be used includes but is not limited to the above-mentioned C_{1-6} alkyl, C_{3-8} cycloalkyl and C_{7-14} aralkyl as well as 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacyl, benzyloxycarbonylhydrazido, tertiary-butoxycarbonylhydrazido, and tritylhydrazido.

The hydroxy group of serine and threonine can be protected by esterification or etherification. The group suited for said esterification includes carbon-derived groups such as lower alkanoyl groups, e.g. acetyl etc., aroyl groups, e.g. benzoyl etc., benzyloxycarbonyl, and ethoxycarbonyl. The group suited for said etherification includes benzyl, tetrahydropyranyl, and tertiary-butyl.

The protective group for the phenolic hydroxyl group of tyrosine includes Bzl, Cl_2-Bzl, 2-nitrobenzyl,

Br-Z, and tertiary-butyl.

The protecting group for imidazole moiety of histidine includes Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, and Fmoc.

The activated carboxyl group of the starting amino acid includes the corresponding acid anhydride, azide, and active esters, e.g. esters with alcohols such as pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccinimide, N-hydroxyphthalimide, HOBt, etc. The activated amino group of the starting amino acid includes the corresponding phosphoramide.

The method for elimination of protective groups includes catalytic reduction using hydrogen gas in the presence of a catalyst such as palladium black or palladium-on-carbon, acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, or a mixture of such acids, base treatment with diisopropylethylamine, triethylamine, piperidine, piperazine, reduction with sodium metal in liquid ammonia. The elimination reaction by the above-mentioned acid treatment is generally carried out at a temperature of -20°C - 40°C and can be conducted advantageously with addition of a cation acceptor such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethyl sulfide, 1,4-butanedithiol, 1,2-ethanedithiol. The 2,4-dinitrophenyl group used for protecting the imidazole group of histidine can be eliminated by treatment with thiophenol, while the formyl group used for protecting the indole group of tryptophan can be eliminated by alkali treatment with dilute sodium hydroxide solution or dilute aqueous ammonia as well as the above-mentioned acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol.

The method for protecting functional groups which should not take part in the reaction of the starting material, the protective groups that can be used, the method of removing the protective groups, and the method of activating the functional groups that are to take part in the reaction can all be selected from among the known groups and methods.

An another method for obtaining the amide form of the polypeptide comprises amidating the α-carboxyl group of the C-terminal amino acid at first, then extending the peptide chain to the N-side until the desired chain length, and then selectively deprotecting the α-amino group of the C-terminal peptide and the α-carboxy group of the amino acid or peptide that is to form the remainder of the objective polypeptide and condensing the two fragments whose α-amino group and side-chain functional groups have been protected with suitable protective groups mentioned above in a mixed solvent such as that mentioned hereinbefore. The parameters of this condensation reaction can be the same as described hereinbefore. From the protected peptide obtained by condensation, all the protective groups are removed by the above-described method to thereby provide the desired crude peptide. This crude peptide can be purified by known purification procedures and the main fraction be lyophilized to provide the objective amidated polypeptide.

To obtain an ester of the polypeptide, the α-carboxyl group of the C-terminal amino acid is condensed with a desired alcohol to give an amino acid ester and then, the procedure described above for production of the amide is followed.

The ligand polypeptide of the present invention, its amide or ester, or a salt thereof can be any peptide that has the same activities, e.g. pituitary function modulating activity, central nervous system

function modulating activity, or pancreatic function modulating activity as the polypeptide which has an amino acid sequence of SEQ ID NO:1 or substantial equivalent thereto. Among such peptides,

(1) Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Thr-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Thr-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe (SEQ ID NO:5) and

(2) Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Thr-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe (SEQ ID NO:6)

are preferable.

The ligand polypeptide or partial peptide thereof can be used as antigen for preparation of anti-ligand polypeptide antibody. The preferable polypeptide as antigen includes

(1) Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu (SEQ ID NO:7),

(2) Thr-Pro-Asp Ile-Asn-Pro-Ala-Trp-Tyr (SEQ ID NO:8) and

(3) Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe (SEQ ID NO:9).

The ligand peptide may be a peptide containing each of the domains which can act as an antigenic determinant or a peptide containing a plurality of the domains within the molecule.

The ligand peptide mentioned in this specification may be one ending with an amide bond ($-CONH_2$) or an ester bond (-COOR) at the C-terminus. The ester here includes the same one of the above polypeptide. When the ligand peptide has a carboxyl or carboxylato group in any position other than the C-terminus, the case in which such group or moiety has been amidated or esterified also falls within the scope of the ligand peptide in the present invention. The ester here may be of the same one as the above-mentioned ester at the C-terminus.

The ligand polypeptide or its partial peptide of the present invention may be in the form of a fused

protein which is fused with a protein whose functions or properties are already known.

The salt of such partial peptide of the ligand polypeptide of present invention may be of the same one as the above-mentioned salt of the polypeptide.

The partial peptide of the ligand polypeptide of the invention, its amide or ester, or a salt thereof can be produced by the same synthetic processes as mentioned for the polypeptide or by cleaving the polypeptide of the present invention with a suitable peptidase.

The DNA coding for the ligand polypeptide or a partial peptide thereof of the present invention may be any DNA comprising the nucleotide sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:1 or substantial equivalent thereto. Furthermore, the DNA may be any of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such a library may be any of bacteriophage, plasmid, cosmid, and phagimid. Moreover, it can be directly amplified by the RT-PCR (reverse transcription polymerase chain reaction) method by using an RNA fraction may be prepared from a tissue or cells.

To be more specific, as the DNA coding for a polypeptide comprising the amino acid sequence of SEQ ID NO:1, the cDNA comprising the nucleotide sequence of SEQ ID NO:2 or the genomic DNA comprising the nucleotide sequence of SEQ ID NO:3 can be exemplified.

Among DNAs coding for the mouse-derived polypeptide comprising the amino acid sequence of SEQ ID NO:1, DNA fragments comprising partial nucleotide sequences of 6 to 90, preferably 6 to 60, more preferably 9 to 30, and especially preferably 12 to 30 can be advantageously used as DNA probes as well.

The DNA coding for the ligand polypeptide of the

present invention can be produced by the following genetic engineering procedures.

The DNA fully encoding the polypeptide or partial peptide of the present invention can be cloned either by PCR amplification using synthetic DNA primers having a partial nucleotide sequence of the polypeptide or partial peptide or by hybridization using the DNA inserted in a suitable vector and labeled with a DNA fragment comprising a part or full region of a murine-derived polypeptide or a synthetic DNA. The hybridization can be carried out typically by the procedure described in Molecular Cloning (2nd ed., J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). When a commercial library is used, the instructions given in the accompanying manual can be followed.

The cloned DNA coding for the polypeptide or partial peptide can be used directly or after digestion with a restriction enzyme or addition of a linker depending on purposes. This DNA has ATG as the translation initiation codon at the 5' end and may have TAA, TGA, or TAG as the termination codon at the 3' end. The translation initiation and termination codons can be added by means of suitable DNA adapters.

An expression vector for the polypeptide or partial peptide can be produced by, for example (a) cutting out a target DNA fragment from the DNA for the polypeptide or partial peptide of the present invention and (b) ligating the target DNA fragment with the downstream side of a promoter in a suitable expression vector.

The vector may include plasmids derived from Escherichia coli, e.g., pBR322, pBR325, pUC12, pUC13, etc.; plasmids derived from Bacillus subtilis, e.g., pUB110, pTP5, pC194, etc.; plasmids derived from yeasts e.g., pSH19, pSH15, etc.; bacteriophages such as λ - phage, and animal viruses such as retrovirus, vaccinia

virus and baculovirus.

According to the present invention, any promoter can be used as long as it is compatible with the host cell which is used for expressing a gene. When the host for the transformation is E. coli, the promoters are preferably trp promoters, lac promoters, recA promoters, λ_{PL} promoters, lpp promoters, etc. When the host for the transformation is Bacillus, the promoters are preferably SPO1 promoters, SPO2 promoters, penP promoters, etc. When the host is a yeast, the promoters are preferably PHO5 promoters, PGK promoters, GAP promoters, ADH promoters, etc. When the host is an animal cell, the promoters include SV40-derived promoters, retrovirus promoters, metallothionein promoters, heat shock promoters, cytomegalovirus (CMV) promoters, SRα promoters, etc. An enhancer can be effectively utilized for expression.

As required, furthermore, a host-compatible signal sequence is added to the N-terminal side of the polypeptide or partial peptide thereof. When the host is E. coli, the utilizable signal sequences may include alkaline phosphatase signal sequence, OmpA signal sequence, etc. When the host is Bacillus, they may include α -amylase signal sequence, subtilisin signal sequence, etc. When the host is a yeast, they may include mating factor α signal sequence, invertase signal sequence, etc. When the host is an animal cell, they may include insulin signal sequence, α-interferon signal sequence, antibody molecule signal sequence, etc.

A transformant or transfectant is produced by using the vector thus constructed, which carries the polypeptide- or partial peptide-encoding DNA of the present invention. The host may be, for example, Escherichia microorganisms, Bacillus microorganisms, yeasts, insect cells, animal cells, etc. Examples of

the Escherichia and Bacillus microorganisms include Escherichia coli K12·DH1 [Proc. Natl. Acad. Sci. USA, Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research, Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology, Vol. 120, 517 (1978)], HB101 [Journal of molecular Biology, Vol, 41, 459 (1969)], C600 [Genetics, Vol. 39, 440 (1954)], etc. Examples of the Bacillus microorganism are, for example Bacillus subtilis MI114 [Gene, Vol. 24, 255 (1983)], 207-21 [Journal of Biochemistry, Vol. 95, 76 (1984)], etc. The yeast may be, for example, Saccharomyces cerevisiae AH22, AH22R⁻, NA87-11A, DKD-5D, 20B-12, etc. The insect may include a silkworm (Bombyx mori larva), [Maeda et al, Nature, Vol. 315, 592 (1985)] etc. The host animal cell may be, for example, monkey-derived cell line, COS-7, Vero, Chinese hamster ovary cell line (CHO cell), DHFR gene-deficient Chinese hamster cell line (dhfr⁻ CHO cell), mouse L cell, mouse myeloma cell, human FL, etc.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Transformation of Escherichia microorganisms can be carried out in accordance with the methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 69, 2110 (1972), Gene, Vol. 17, 107 (1982), etc. Transformation of Bacillus microorganisms can be carried out in accordance with the methods as disclosed in, for example, Molecular & General Genetics, Vol. 168, 111 (1979), etc. Transformation of the yeast can be carried out in accordance with the methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 75, 1929 (1978), etc. The insect cells can be transformed in accordance with the methods as disclosed in, for example, Bio/Technology, 6, 47-55, 1988. The animal cells can be transformed by the methods as disclosed in, for example, Virology, Vol. 52, 456,

1973, etc. The transformants or transfectants which harbor the expression vector carrying DNA encoding a polypeptide or partial peptide thereof are produced according to the aforementioned techniques.

Cultivation of the transformant (transfectant) in which the host is Escherichia or Bacillus microorganism can be carried out suitably in a liquid culture medium. The culture medium may contains carbon sources, nitrogen sources, minerals, etc. necessary for growing the transformant. The carbon source may include glucose, dextrin, soluble starch, sucrose, etc. The nitrogen source may include organic or inorganic substances such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, bean-cakes, potato extracts, etc. Examples of the minerals may include calcium chloride, sodium dihydrogen phosphate, magnesium chloride, etc. It is further allowable to add yeast extracts, vitamines, growth-promoting factors, etc. It is desired that the culture medium is pH from about 5 to about 8.

The Escherichia microorganism culture medium is preferably an M9 medium containing, for example, glucose and casamino acids (Miller, Journal of Experiments in Molecular Genetics), 431-433, Cold Spring Harbor Laboratory, New York, 1972. Depending on necessity, the medium may be supplemented with drugs such as 3β -indolyl acrylic acid in order to improve efficiency of the promoter. In the case of an Escherichia host, the cultivation is carried out usually at about 15 to 43°C for about 3 to 24 hours. As required, aeration and stirring may be applied. In the case of Bacillus host, the cultivation is carried out usually at about 30 to 40°C for about 6 to 24 hours. As required, aeration and stirring may be also applied. In the case of the transformant in which the host is a yeast, the culture medium used may include,

for example, a Burkholder minimum medium [Bostian, K.L. et al., Proc. Natl. Acad. Sci. USA, Vol. 77, 4505 (1980)], an SD medium containing 0.5% casamino acids [Bitter, G.A. et al., Proc. Natl. Acad. Sci. USA, Vol. 81, 5330 (1984)], etc. It is preferable that the pH of the culture medium is adjusted to be from about 5 to about 8. The cultivation is carried out usually at about 20 to 35°C for about 24 to 72 hours. As required, aeration and stirring may be applied. In the case of the transformant in which the host is an insect, the culture medium used may include those obtained by suitably adding additives such as passivated (or immobilized) 10% bovine serum and the like to the Grace's insect medium (Grace, T.C.C., Nature, 195, 788 (1962)). It is preferable that the pH of the culture medium is adjusted to be about 6.2 to 6.4. The cultivation is usually carried out at about 27°C for about 3 to 5 days. As desired, aeration and stirring may be applied. In the case of the transformant in which the host is an animal cell, the culture medium used may include MEM medium [Science, Vol. 122, 501 (1952)], DMEM medium [Virology, Vol. 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association, Vol. 199, 519 (1967)], 199 medium [Proceedings of the Society of the Biological Medicine, Vol. 73, 1 (1950)], etc. which are containing, for example, about 5 to 20% of fetal calf serum. It is preferable that the pH is from about 6 to about 8. The cultivation is usually carried out at about 30 to 40°C for about 15 to 60 hours. As required, medium exchange, aeration and stirring may be applied.

Separation and purification of the polypeptide or partial peptide from the above-mentioned cultures can be carried out according to methods described herein below.

To extract the polypeptide or partial peptide from

the cultured microorganisms or cells, the microorganisms or cells are collected by known methods after the cultivation, suspended in a suitable buffer solution, disrupted by ultrasonic waves, lysozyme and/or freezing and thawing, etc. and, then, a crude extract of the polypeptide or partial peptide is obtained by centrifugation or filtration. Other conventional extracting or isolating methods can be applied. The buffer solution may contain a protein-denaturing agent such as urea or guanidine hydrochloride or a surfactant such as Triton X-100 (registered trademark, hereinafter often referred to as "TM").

In the case where the polypeptide or partial peptide is secreted into culture media, supernatant liquid is separated from the microorganisms or cells after the cultivation is finished and the resulting supernatant liquid is collected by widely known methods. The culture supernatant liquid and extract containing the polypeptide or partial peptide can be purified by a suitable combination of widely known methods for separation, isolation and purification. The widely known methods of separation, isolation and purification may include methods which utilizes solubility, such as salting out or sedimentation with solvents, methods which utilizes chiefly a difference in the molecular size or weight, such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in the electric charge, such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in the hydrophobic property, such as reverse-phase high-performance liquid chromatography, and methods utilizing a difference in the isoelectric point such as isoelectric electrophoresis, or

chromatofocusing, etc.

In cases where the polypeptide or partial peptide thus obtained is in a free form, the free protein can be converted into a salt thereof by known methods or method analogous thereto. In case where the polypeptide or partial peptide thus obtained is in a salt form vice versa, the protein salt can be converted into a free form or into any other salt thereof by known methods or method analogous thereto.

The polypeptide or partial peptide produced by the transformant can be arbitrarily modified or a polypeptide can be partly removed therefrom, by the action of a suitable protein-modifying enzyme before or after the purification. The protein-modifying enzyme may include trypsin, chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase, etc. The activity of the polypeptide or partial peptide thus formed can be measured by experimenting the coupling (or binding) with receptor or by enzyme immunoassays (enzyme linked immunoassays) using specific antibodies.

The DNA coding for the ligand polypeptide of the present invention, the ligand polypeptide or a partial peptide thereof can be used for (1) synthesis of a part or the full length of the ligand for G protein-coupled receptor protein, (2) search for the physiological activities of the ligand polypeptide or partial peptide thereof of the present invention, (3) preparation of a synthetic oligonucleotide probe or a PCR primer, (4) acquisition of DNAs coding for ligands of G protein-coupled receptor proteins and precursor proteins, (5) development of receptor-binding assay systems using the expression of recombinant receptor proteins and screening of candidates for medicinally active compounds, (6) acquisition of antibodies and antisera, (7) development of diagnostic agents utilizing said antibodies or antisera, (8) development of drugs such

as pituitary function modulators, central nervous system function modulators, and pancreatic function modulators, and (9) gene therapies, among other uses.

Particularly the DNA comprising the DNA cording the ligand polypeptide of this invention is useful for a production of a non-human transgenic animal or a knock out mouse and for analyzing a physiological function or a functional mechanism of the ligand polypeptide by using the non-human transgenic animal or the knock out mouse.

Further, referring to (8) above, the ligand polypeptide, or the DNA encording either of them of the present invention is useful as a safe pharmaceutical composition of low toxic potential because it is recognized as a ligand by the G protein-coupled receptor protein expressed in the hypophysis, central nervous system and pancreatic β cells. The ligand polypeptide, a partial peptide thereof, or the DNA encoding either of them of the present invention is associated with the modulation of pituitary function, central nervous system function, and pancreatic function and, therefore, can be used as a pharmaceutical composition for treatment or prevention of dementia such as senile dementia, cerebrovascular dementia (dementia due to cerebrovascular disorder), dementia associated with phylodegenerative retroplastic diseases (e.g. Alzheimer's disease, Parkinson's disease, Pick's disease, Huntington's disease, etc.), dementia due to infectious diseases (e.g. delayed viral infections such as Creutzfelt-Jakob disease), dementia associated with endocrine, metabolic, and toxic diseases (e.g. hypothyroidism, vitamin B12 deficiency, alcoholism, and poisoning due to various drugs, metals, or organic compounds), dementia associated with oncogenous diseases (e.g. brain tumor), dementia due to traumatic diseases (e.g. chronic subdural hematoma):,

depression (melancholia), hyperkinetic (microencephalopathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease (e.g. gigantism, acromegalic gigantism etc.), hyperphagia, polyphagia, hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, diabetes (e.g. diabetic complications, diabetic nephropathy, diabetic neurophathy, diabetic retinopathy etc.), cancer (e.g. mammary cancer, lymphatic leukemia, cystic cancer, ovary cancer, prostatic cancer etc.), pancreatitis, renal disease (e.g. chromic renal failure, nephritis etc.), Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility or oligogalactia. Furthermore, they can be also used as the agent for improvement in postoperative nutritional status and/or vasopressor.

When the polypeptide, or the DNA encoding either of them of the present invention is used as a pharmaceutical composition as described above, it can be used by conventional methods. For example, it can be used orally in the form of tablets which may be sugar coated as necessary, capsules, elixirs, microcapsules etc., or non-orally in the form of injectable preparations such as aseptic solutions and suspensions in water or other pharmaceutically acceptable liquids. These preparations can be produced by mixing the polypeptide, a partial peptide thereof, or the DNA encoding either of them with physiologically acceptable carriers, flavoring agents, excipients, vehicles, antiseptics, stabilizers, binders etc. in unit dosage forms required for generally accepted

manners of pharmaceutical manufacture. Active ingredient contents in these preparations are set so that an appropriate dose within the specified range is obtained.

Additives which can be mixed in tablets, capsules etc. include binders such as gelatin, corn starch, tragacanth and gum arabic, excipients such as crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweetening agents such as sucrose, lactose and saccharin, and flavoring agents such as peppermint, akamono oil and cherry. When the unit dosage form is a capsule, the above-mentioned materials may further incorporate liquid carriers such as oils and fats. Sterile compositions for injection can be formulated by ordinary methods of pharmaceutical manufacture, for example, by dissolving or suspending active ingredients, naturally occuring vegetable oils such as sesame oil and coconut oil, etc. in vehicles such as water for injection.

Aqueous liquids for injection include physiological saline and isotonic solutions containing glucose and other auxiliary agents, e.g., D-sorbitol, D-mannitol and sodium chloride, and may be used in combination with appropriate dissolution aids such as alcohols, e.g., ethanol, polyalcohols, e.g., propylene glycol and polyethylene glycol, nonionic surfactants, e.g., polysorbate 80 (TM) and HCO-50 etc. Oily liquids include sesame oil and soybean oil, and may be used in combination with dissolution aids such as benzyl benzoate and benzyl alcohol. Furthermore the above-mentioned materials may also be formulated with buffers, e.g., phosphate buffer and sodium acetate buffer; soothing agents, e.g., benzalkonium chloride, procaine hydrochloride; stabilizers, e.g., human serum albumin, polyethylene glycol; preservatives, e.g.,

benzyl alcohol, phenol; antioxidants etc. The thus-prepared injectable liquid is normally filled in an appropriate ampule. Because the thus-obtained preparation is safe and of low toxicity, it can be administered to humans or warm-blooded mammals, e.g., mouse, rats, guinea pig, rabbits, chicken, sheep, pigs, bovines, cats, dogs, monkeys, baboons, chimpanzees, for instance.

The dose of said polypeptide, or the DNA encoding either of them is normally about 0.1-100 mg, preferably 1.0-50 mg, and more preferably 1.0-20 mg per day for an adult (weighing 60 kg) in oral administration, depending on symptoms etc. In non-oral administration, it is advantageous to administer the polypeptide, a partial peptide thereof, or the DNA encoding either of them in the form of injectable preparation at a daily dose of about 0.01-30 mg, preferably about 0.1-20 mg, and more preferably about 0.1-10 mg per administration by an intravenous injection for an adult (weighing 60 kg), depending on subject of administration, target organ, symptoms, method of administration etc. For other animal species, corresponding does as converted per 60 kg weight can be administered.

The G protein-coupled receptor protein for the above ligand polypeptide of the present invention may be any of G protein-coupled receptor proteins derived from various tissues, e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow, adrenal gland, skin, muscle, lung, alimentary canal, blood vessel, heart, etc. of human and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.; and comprising an amino acid sequence of SEQ ID NO:10, 11, 12, 18 or 14, or a substantial equivalent thereto. Thus, the G protein-coupled receptor protein of the present invention includes, in addition to a protein comprising

the SEQ ID NO:10, 11, 12, 13 or 14, those proteins comprising amino acid sequences of about 90-99.9% homology to the amino acid sequence of SEQ ID NO:10, 11, 12, 13 or 14 and having qualitatively substantially equivalent activity to proteins comprising the amino acid sequence of SEQ ID NO:10, 11, 12, 13, or 14. The activities which these proteins are possessed may include ligand binding activity and signal transduction activity. The term "substantially equivalent" means that the nature of the ligand binding activity and the like is equivalent. Therefore, it is allowable that even differences among grades such as the strength of ligand binding activity and the molecular weight of receptor protein are present.

To be further specific, the G protein-coupled receptor proteins include human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:10 or/and SEQ ID NO:11, mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:13, and mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:14. As the human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:10 and/or SEQ ID NO:11 include the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:12.

Here, the protein which comprises an amino acid sequence of SEQ ID NO:12 or a substantial equivalent thereto contains the full-length of the amino acid sequence for human pituitary-derived G protein-coupled receptor protein. The protein which comprises an amino acid sequence of SEQ ID NO:10 or/and SEQ ID NO:11 or a substantial equivalent thereto may be a partial peptide of the protein which comprises an amino acid sequence

of SEQ ID NO:12 or a substantial equivalent thereto. The protein which comprises an amino acid sequence of SEQ ID NO:13 or SEQ ID NO:14 or a substantial equivalent thereto is a G protein-coupled receptor protein which is derived from mouse pancreas but, since its amino acid sequence is quite similar to the amino acid sequence of SEQ ID NO:10 or/and SEQ ID NO:11, the protein which comprises an amino acid sequence of SEQ ID NO:13 or 14 or a substantial equivalent thereto is also subsumed in the category of said partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:12 or a substantial equivalent thereto.

Thus, the above-mentioned protein comprising an amino acid sequence of SEQ ID NO:12 or a substantial equivalent thereto or a partial peptide of the protein or a salt thereof, which will be described below, includes the protein comprising an amino acid sequence of SEQ ID NO:10, 11, 12, or 13 or a substantial equivalent thereto, or a salt thereof.

Furthermore, the G protein-coupled receptor protein includes the protein in which the N-terminal Met has been protected with a protective group, e.g. C_{1-6} acyl such as formyl or acetyl, the protein in which the N-terminal side of Gln has been cleaved in vivo to form pyroglutamyl, the protein in which the side chain of any relevant constituent amino acid has been protected with a suitable protective group, e.g. C_{1-6} acyl such as formyl or acetyl, and the complex protein such as glycoproteins available upon attachment of sugar chains.

The salt of G protein-coupled receptor protein includes the same kinds of salts as mentioned for the ligand polypeptide.

The G protein-coupled receptor protein or a salt thereof or a partial peptide thereof can be produced from the tissues or cells of human or other warm-

blooded animals by per se known purification techniques or, as described above, by culturing a transformant carrying a DNA coding for the G protein-coupled receptor protein. It can also be produced in accordance with the procedures for peptide synthesis which are described above. The production method is described in, for example, Examples 3, 4, 6 and 17 of WO96/05302, in detail.

The DNA coding for the G protein-coupled receptor protein may be any DNA comprising a nucleotide sequence encoding the G protein-coupled receptor protein which comprises an amino acid sequence of SEQ ID NO:10, 11, 12, 13, or 14 or a substantial equivalent thereto. It may also be any one of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such a library may include bacteriophage, plasmid, cosmid, and phargimid. Furthermore, using an RNA fraction prepared from a tissue or cells, a direct amplification can be carried out by the RT-PCR method.

To be specific, the DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:10 include a DNA which comprises the nucleotide sequence of SEQ ID NO:15. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:11 include a DNA which comprises the nucleotide sequence of SEQ ID NO:16. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:12 include a DNA which comprises the nucleotide sequence of SEQ ID NO:17. The DNA encoding the mouse pancreas-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:13 include a DNA which comprises the nucleotide sequence

of SEQ ID NO:18. The DNA encoding the mouse pancreas-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:14 include a DNA comprising the nucleotide sequence of SEQ ID NO:19.

A method for cloning the DNA completely coding for the G protein-coupled receptor protein, vector, promoter, host cell, a method for transformation, a method for culturing the transformant or a method for separation and purification of the G protein-coupled receptor protein may include the same one as mentioned for the ligand polypeptide.

Described below are uses of the ligand polypeptide of the present invention, the G protein-coupled receptor protein-encoding DNAs and their antibodies.

(1) Prophylactic and Therapeutic Agent for Ligand Polypeptide Deficiency Diseases

The G protein-coupled receptor protein-encoding DNA can be used as a prophylactic and/or therapeutic agent for treating said ligand polypeptide deficiency diseases depending upon the action that said ligand exerts.

For example, when there is a patient for whom the physiological action of the ligand, such as pituitary function modulating action, central nervous system function modulating action or pancreatic function modulating action, cannot be expected because of a descrease in the G protein-coupled receptor protein or ligand polypeptide in vivo, the amount of the G protein-coupled receptor protein or ligand polypeptide in the brain cells of said patient can be increased whereby the action of the ligand can be fully achieved by:

(a) administering the G protein-coupled receptor protein-encoding DNA to the patient to express it; or

(b) inserting the G protein-coupled receptor protein

or ligand polypeptide-encoding DNA into brain cells or the like to said patient. Accordingly, the G protein-coupled receptor protein- or ligand polypeptide-encoding DNA can be used as a safe and less toxic preventive and therapeutic agent for the G protein-coupled receptor protein or ligand polypeptide deficiency diseases.

When the above-mentioned DNA is used as the above-mentioned agent, said DNA may be used alone or after inserting it into a suitable vector such as retrovirus vector, adenovirus vector, adenovirus-associated virus vector, etc. followed by subjecting the product vector to a conventional means which is the same means as using the DNA coding for the ligand polypeptide or partial peptide thereof as the pharmaceutical composition.

(2) Quantitative Determination of the G protein-coupled receptor Protein to the Ligand Polypeptide

The ligand polypeptide that has a binding property for a G protein-coupled receptor protein or a partial peptide thereof, or a salt thereof is capable of determining quantitatively an amount of a G protein-coupled receptor protein or a partial peptide thereof, or a salt thereof in vivo with good sensitivity.

This quantitative determination may be carried out by, for example, combining with a competitive analysis. Thus, a sample to be determined is contacted with the ligand polypeptide so that the concentration of a G protein-coupled receptor protein or a partial peptide thereof in said sample can be determined. In one embodiment of the quantitative determination, the protocols described in the following 1) and 2) or methods similar thereto may be used:

1) Hiroshi Irie (ed): "Radioimmunoassay" (Kodansha, Japan, 1974); and

2) Hiroshi Irie (ed): "Radioimmunoassay, Second

Series" (Kodansha, Japan, 1979).

(3) Screening of Compound Changing the Binding Activity of Ligand Polypeptide, or salt thereof (hereinafter sometimes referred to briefly as ligand or ligand polypeptide) with the G protein-coupled receptor Protein

G protein-coupled receptor proteins or partial peptide or salt thereof can be used. Alternatively, expression systems for recombinant G protein-coupled receptor proteins are constructed and receptor binding assay systems using said expression system are used. In these assay systems, it is possible to screen compounds, e.g. peptides, proteins, nonpeptidic compounds, synthetic compounds, formentation products, cell extracts, animal tissue extracts, etc.; or salts thereof which change the binding activity of a ligand polypeptide with the G protein-coupled receptor protein. Such a compound includes a compound exhibiting a G protein-coupled receptor-mediated cell stimulating activity, e.g. activity of promoting or activity of inhibiting physiological reactions including liberation of arachidonic acid, liberation of acetylchloline, intracellular Ca^{2+} liberation, intracellular cAMP production, intracellular cGMP production, production of inositol phosphate, changes in cell membrane potential, phosphorylation of intracellular proteins activation of c-fos, lowering of pH, activation of G protein, cell promulgation, etc.; so-called "G protein-coupled receptor-agonist", a compound free from such a cell stimulating activity, so-called "G protein coupled receptor-antagonist", etc. The term of "change the binding activity of a ligand polypeptide" includes the both concept of the case in which the binding of ligand is inhibited and the case in which the binding of ligand is promoted.

Thus, the present invention provides a method of

screening for a compound which changes the binding activity of a ligand with a G protein-coupled receptor protein or a salt thereof, characterized by comparing the following two cases:

(i) the case wherein the ligand is contacted with the G protein-coupled receptor protein or salt thereof, or the partial peptide thereof or a salt thereof; and

(ii) the case wherein the ligand is contacted with a mixture of the G protein-coupled receptor protein or salt thereof or the partial peptide or salt thereof and said test compound.

In said screening method, one characteristic feature of the present invention resides in that the amount of the ligand bonded with said G protein-coupled receptor protein or the partial peptide thereof, the cell stimulating activity of the ligand, etc. are measured in both the case where (i) the ligand polypeptide is contacted with G protein-coupled receptor proteins or partial peptide thereof and in the case where (ii) the ligand polypeptide and the test compound are contacted with the G protein-coupled receptor protein or the partial peptide thereof, respectively and then compared therebetween.

In one more specific embodiment of the present invention, the following is provided:

1) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with a G protein-coupled receptor protein or a partial peptide thereof and when a labeled ligand polypeptide and a test compound are contacted with a G protein-coupled receptor protein or a partial peptide thereof, the amounts of the labeled ligand polypeptide bonded with said protein or a partial peptide thereof or a salt thereof are measured

and compared;

2) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with cells containing G protein-coupled receptor proteins or a membrane fraction of said cells and when a labeled ligand polypeptide and a test compound are contacted with cells containing G protein-coupled receptor proteins or a membrane fraction of said cells, the amounts of the labeled ligand polypeptide binding with said protein or a partial peptide thereof or a salt thereof are measued and compared;

3) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a labeled ligand polypeptide is contacted with G protein-coupled receptor proteins expressed on the cell memberane by culturing a transformant carrying a G protein-coupled receptor protein-encoding DNA and when a labeled ligand polypeptide and a test compound are contacted with G protein-coupled receptor proteins expressed on the cell membrane by culturing a transformant carrying a G protein-coupled receptor protein-encoding DNA, the amounts of the labeled ligand polypeptide binding with said G protein-coupled receptor protein are measured and compared;

4) a method of screening for a compound or a salt thereof which changes the binding of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a G protein-coupled receptor protein-activating compound, e.g. a ligand polypeptide of the present invention, etc. is contacted with cells containing G protein-coupled receptor

proteins and when the G protein-coupled receptor protein-activating compound and a test compound are contacted with cells containing G protein-coupled receptor proteins, the resulting G protein-coupled receptor protein-mediated cell stimulting activities, e.g. activities of promoting or activities of inhibiting physiological responses including liberation of arachidonic acid, liberation of acetylcholine, intracellular Ca^{2+} liberation, intracellular cAMP production, intracellular cGMP production, production of inositol phosphate, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, lowering of pH, activation of G protein, cell promulgation, etc.; are measured and compared; and

5) a method of screening for a compound or a salt thereof which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, characterized in that, when a G protein-coupled receptor protein-activating compound, e.g. a ligand polypeptide of the present invention, etc. is contacted with G protein-coupled receptor proteins expressed on cell membranes by culturing transformants carrying G protein-coupled receptor protein-encoding DNA and when a G protein-coupled receptor protein-activating compound and a test compound are contacted with the G protein-coupled receptor protein expressed on the cell membrane by culturing the transformant carrying the G protein-coupled receptor protein-encoding DNA, the resulting G protein-coupled receptor protein-mediated cell stimulating activities, e.g. activities of promoting or activities of inhibiting physiological responses such as liberation of arachidonic acid, liberation of acetylcholine, intracellular Ca^{2+} liberation, intracellular cAMP production, intracellular cGMP production, production of inositol

phosphate, changes in cell membrane potential, phosphorylation of intracellular proteins, activation of c-fos, lowering of pH, activation of G protein, and cell promulgation, etc.; are measured and compared.

The G protein-coupled receptor agonist or antagonist can be screened by, first, obtaining a candidate compound by using G protein-coupled receptor protein-containing cells, tissues or cell membrane fractions derived from mouse, rat or the like (primary screening), then, making sure whether the candidate compound really inhibits the binding between human G protein-coupled receptor proteins and ligands (secondary screening). Other receptor proteins inevitably exist and when the cells, the tissues or the cell membrane fractions are used, they intrinsically make it difficult to screen agonists or antagonists to the desired receptor proteins. By using the human-derived G protein-coupled receptor protein, however, there is no need of effecting the primary screening, whereby it is possible to efficiently screen a compound that changes the binding activity between a ligand and a G protein-coupled receptor. Additionally, it is possible to evaluate whether the compound that is screened is a G protein-coupled receptor agonist or a G protein-coupled receptor antagonist.

Specific explanations of the screening method will be given as hereunder.

First, with respect to the G protein-coupled receptor protein used for the screening method of the present invention, any product may be used so far as it contains G protein-coupled receptor proteins or partial peptides thereof although the use of a membrane fraction of mammalian organs is preferable. However, human organs can be extremely scarce and, accordingly, G protein-coupled receptor proteins which are expressed in a large amount using a recombinant technique are

suitable for the screening.

In the manufacture of the G protein-coupled receptor protein, the above-mentioned method can be used.

When the G protein-coupled receptor protein-containing cells or cell membrane fractions are used in the screening method, the above-mentioned method can be used.

In conducting the above-mentioned methods 1) to 3) for screening the compound capable of changing the binding activity of the ligand with the G protein-coupled receptor protein, a suitable G protein-coupled receptor fraction and a labeled ligand polypeptide are necessary. With respect to the G protein-coupled receptor fraction, it is preferred to use naturally occurring G protein-coupled receptors (natural type G protein-coupled receptors) or recombinant type G protein-coupled receptor fractions with the activity equivalent to that of the natural type G protein coupled reaction. Here the term "activity equivalent to" means the same ligand binding activity, or the substantially equivalent ligand binding activity.

With respect to the labeled ligand, it is possible to use labeled ligands, labeled ligand amalogized compounds, etc. For example, ligands labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc. and other labeled substances may be utilized.

Specifically, G protein-coupled receptor protein-containing cells or cell membrane fractions are first suspended in a buffer which is suitable for the determining method to prepare the receptor sample in conducting the screening for a compound which changes the binding activity of the ligand with the G protein-coupled receptor protein. With respect to the buffer, any buffer such as Tris-HCl buffer or phosphate buffer of pH 4-10, preferably, pH 6-8 which does not inhibit

the binding of the ligand with the receptor may be used.

In addition, a surface-active agent such as CHAPS, Tween 80™ (Kao-Atlas, Japan), digitonin, deoxycholate, etc. and/or various proteins such as bovine serum albumin (BSA), gelatin, etc. may be added to the buffer with an object of decreasing the nonspecific binding. Further, a protease inhibitor such as PMSF, leupeptin, E-64 manufactured by Peptide Laboratory, Japan, pepstatin, etc. may be added with an object of inhibiting the decomposition of the receptor and the ligand by protease. A labeled ligand in a certain amount (5,000 cpm to 500,000 cpm) is added to 0.01 ml to 10 ml of said receptor solution and, at the same time, 10^{-4}M to 10^{-10}M of a test compound coexists. In order to determine the nonspecific binding amount (NSB), a reaction tube to which a great excessive amount of an unlabeled test compounds is added is prepared as well.

The reaction is carried out at 0-50°C, preferably at 4-37°C for 20 minutes to 24 hours, preferably 30 minutes to three hours. After the reaction, it is filtered through a glass fiber filter, a filter paper, or the like, washed with a suitable amount of the same buffer and the radioactivity retained in the glass fiber filter, etc. is measured by means of a liquid scintillation counter of a gamma-counter. Supposing that the count (B_0 - NSB) obtained by subtracting the nonspecific binding amount (NSB) from the total binding amount (B_0) wherein an antagonizing substance is not present is set at 100%, a test compound in which the specific binding amount (B - NSB) obtained by subtracting the nonspecific binding amount (NSB) from the total binding amount (B) is, for example, less than 50% may be selected as a candidate ligand to the G protein-coupled receptor protein of the present

invention.

In conducting the above-mentioned methods 4) to 5) for screening the compound which changes the binding activity of the ligand with the G protein-coupled receptor protein, the G protein-coupled receptor protein-mediated cell stimulating activity, e.g. activities of promoting or activities of inhibiting physiological responses such as release of arachidonic acid, release of acetylcholine, intracellular Ca^{2+} increase, intracellular cAMP production, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of intracullular proteins, activation of c-fos, lowering of pH, activation of G protein and cell proliferation, etc.; may be measured by known methods or by the use of commercially available measuring kits. To be more specific, G protein-coupled receptor protein-containing cells are at first cultured in a multiwell plate or the like.

In conducting the screening, it is substituted with a suitable buffer which does not show toxicity to fresh media or cells in advance, incubated under appropriate conditions and for a specified time after additing a test compound, etc. thereto. The resultant cells are extracted or the supernatant liquid is recovered and the resulting product is determined, preferably quantitatively, by each of the methods. When it is difficult to identify the production of the indicative substance, e.g. arachidonic acid, etc. which is to be an indication for the cell stimulating activity due to the presence of decomposing enzymes contained in the cell, an assay may be carried out by adding an inhibitor against said decomposing enzyme. With respect to the activities such as an inhibitory action against cAMP production, it may be detected as an inhibitory action against the cAMP production in the cells whose fundamental production has been increased

by forskolin or the like.

In conducting a screening by measuring the cell stimulating activity, cells in which a suitable G protein-coupled receptor protein is expressed are necessary. Preferred G protein-coupled receptor protein-expressing cells are naturally occurring G protein-coupled receptor protein (natural type G protein-coupled receptor protein)-containing cell lines or strains, e.g. mouse pancreatic β cell line, MIN6, etc., the above-mentioned recombinant type G protein-coupled receptor protein-expressing cell lines or strains, etc.

Examples of the test compound includes peptide, proteins, non-peptidic compounds, synthesized compounds, fermentation products, cell extracts, plant extracts, animal tissue extracts, serum, blood, body fluid, etc. Those compounds may be novel or known.

A kit for screening the compound which changes the binding activity of the ligand with the G protein-coupled receptor protein or a salt thereof comprises a G protein-coupled receptor protein or a partial peptide thereof, or G protein-coupled receptor protein-containing cells or cell membrane fraction thereof.

Examples of the screening kit include as follows:

1. Reagent for Determining Ligand.

1) Buffer for Measurement and Buffer for Washing.

The product wherein 0.05% of bovine serum albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

This may be sterilized by filtration through a membrane filter with a 0.45 μm pore size, and stored at 4°C or may be prepared upon use.

2) Sample of G protein-coupled receptor Protein.

CHO cells in which a G protein-coupled receptor protein is expressed are subcultured at the rate of 5 x 10^5 cells/well in a 12-well plate and cultured at 37°C

with a 5% CO_2 and 95% air atmosphere for two days to prepare the sample.

3) Labeled Ligand.

The ligand which is labeled with commercially available [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc.

The product in a state of an aqueous solution is stored at 4°C or at -20°C and, upon use, diluted to 1 μM with a buffer for the measurement.

4) Standard Ligand Solution.

Ligand is dissolved in PBS containing 0.1% of bovine serum albumin (manufactured by Sigma) to make 1 mM and stored at -20°C.

2. Method of the Measurement.

1) CHO cells are cultured in a 12-well tissue culture plate to express G protein-coupled receptor proteins. The G protein-coupled receptor protein-expressing CHO cells are washed with 1 ml of buffer for the measurement twice. Then 490 μl of buffer for the measurement is added to each well.

2) Five μl of a test compound solution of 10^{-3} to 10^{-10} M is added, then 5 μl of a labeled ligand is added and subjected to reaction at room temperature for one hour. For knowing the non-specific binding amount, 5 μl of the ligand of 10^{-3} M is added instead of the test compound.

3) The reaction solution is removed from the well, which is washed with 1 ml of buffer for the measurement three times. The labeled ligand binding with the cells is dissolved in 0.2N NaOH-1% SDS and mixed with 4 ml of a liquid scintillator A (such as manufactured by Wako Pure Chemical, Japan).

4) Radioactivity is measured using a liquid scintillation counter (e.g., one manufactured by Beckmann) and PMB (percent maximum binding) is calculated by the following equation:

$$PMB = [(B - NSB)/(B_0 - NSB)] \times 100$$

PMB: Percent maximum binding
B: Value when a sample is added
NSB: Nonspecific binding
B_0: Maximum binding

The compound or a salt thereof obtained by the screening method or by the screening kit is a compound which changes the binding activity of a ligand polypeptide with a G protein-coupled receptor protein, wherein the compound inhibits or promotes the binding, and, more particularly, it is a compound having a cell stimulating activity mediated via a G protein-coupled receptor or a salt thereof, so-called "G protein-coupled receptor agonist" or a compound having no said stimulating activity, so-called "G protein-coupled receptor antagonist". Examples of said compound are peptides, proteins, non-peptidic compounds, synthesized compounds, fermentation products, etc. and the compound may be novel or known.

Said G protein coupled receptor agonist has the same physiological action as the ligand to the G protein-coupled receptor protein and, therefore, it is useful as a safe and less toxic pharmaceutical composition depending upon said ligand activity.

On the other hand, said G protein-coupled receptor antagonist is capable of inhibiting the physiological activity of the ligand to the G protein-coupled receptor protein and, therefore, it is useful as a safe and less toxic pharmaceutical composition for inhibiting said ligand activity.

The ligand polypeptide of the present invention relates to the pituitary function modulating action, central nervous system function modulating action or pancreatic function modulating action. Therefore, the above-mentioned agonist or antagonist can be used as a

therapeutic and/or prophylactic agent for dementia such as senile dementia, cerebrovascular dementia (dementia due to cerebrovascular disorder), dementia associated with phylodegenerative retroplastic diseases (e.g. Alzheimer's disease, Parkinson's disease, Pick's disease, Huntington's disease, etc.), dementia due to infectious diseases (e.g. delayed viral infections such as Creutzfelt-Jakob disease), dementia associated with endocrine, metabolic, and toxic diseases (e.g. hypothyroidism, vitamin B12 deficiency, alcoholism, and poisoning due to various drugs, metals, or organic compounds), dementia associated with oncogenous diseases (e.g. brain tumor), dementia due to traumatic diseases (e.g. chronic subdural hematoma):, depression (melancholia), hyperkinetic (microencephalo-pathy) syndrome, disturbance of consciousness, anxiety syndrome, schizophrenia, horror, growth hormone secretory disease (e.g. gigantism, acromegalic gigantism etc.), hyperphagia, polyphagia, hypercholesterolemia, hyperglyceridemia, hyperlipemia, hyperprolactinemia, hypoglycemia, pituitarism, pituitary drawfism, diabetes (e.g. diabetic complications, diabetic nephropathy, diabetic neurophathy, diabetic retinopathy etc.), cancer (e.g. mammary cancer, lymphatic leukemia, cystic cancer, ovary cancer, prostatic cancer etc.), pancreatitis, renal disease (e.g. chromic renal failure, nephritis etc.), Turner's syndrome, neurosis, rheumatoid arthritis, spinal injury, transient brain ischemia, amyotrophic lateral sclerosis, acute myocardial infarction, spinocerebellar degeneration, bone fracture, trauma, atopic dermatitis, osteoporosis, asthma, epilepsy, infertility or oligogalactia. Furthermore, the agonist or antagonist can be also used as hypnotic-sedative agent for improvement in postoperative nutritional status, vasopressor or

depressor.

When the compound or the salt thereof obtained by the screening method or by the screening kit is used as the pharmaceutical composition, a conventional means which is the same means as using above-mentioned ligand polypeptide as the pharmaceutical compoisiton may be applied therefor.

(4) Manufacture of Antibody or Antiserum against the Ligand Polypeptide of the present invention

Antibodies, e.g. polyclonal antibody, monoclonal antibody, and antisera against the ligand polypeptide may be manufactured by antibody- or antiserum-manufacturing methods per se known to those of skill in the art or methods similar thereto, using the ligand polypeptide as antigen. For example, polyclonal antibodies can be manufactured by the method as given below.

[Preparation of a polyclonal antibody]

The above-mentioned polypeptide or protein as the antigen is coupled to a carrier protein. The carrier protein may for example be bovine thyroglobulin, bovine serum albumin, bovine gamma-globulin, hemocyanine, or Freund's complete adjuvant (Difco).

The coupling reaction between the antigen protein and the carrier protein can be carried out by the known procedure. The reagent for use in the coupling reaction includes but is not limited to glutaraldehyde and water-soluble carbodiimide. The suitable ratio of the antigen protein to the carrier protein is about 1:1 through about 1:10 and as to the reaction pH, satisfactory results are obtained in many cases when the reaction is carried out around neutral, particularly in the range of pH about 6-8. The reaction time is preferably about 1 to 12 hours in many cases and more preferably about 2 to 6 hours. The

conjugate thus obtained is dialyzed against water at about 0 to 18°C in the routine manner and stored frozen or optionally lyophilized and stored.

For the production of a polyclonal antibody, a warm-blooded animal is inoculated with the immunogen produced in the manner described hereinbefore. The warm-blooded animal that can be used for this purpose includes mammalian warm-blooded animals, e.g. rabbit, sheep, goat, rat, mouse, guinea pig, bovine, equine, swine, etc.; and avian species, e.g. chicken, dove, duck, goose, quail, etc. Regarding the methodology for inoculating a warm-blooded animal with the immunogen, the inoculum size of the immunogen may be just sufficient for antibody production. For example, the desired antibody can be produced in many instances by emulsifying 1 mg of the immunogen in 1 ml of saline with Freund's complete adjuvant and injecting the emulsion subcutaneously at the back and hind-limb footpad of rabbits 5 times at 4-week intervals. For harvesting the antibody produced in the warm-blooded animal, for example a rabbit, the blood is withdrawn from the auricular vein usually during day 7 through day 12 after the last inoculation dose and centrifuged to recover an antiserum. For purification, the antiserum is generally subjected to affinity chromatography using a carrier to which each antigen peptide has been conjugated and the adsorbed fraction is recovered to provide a polyclonal antibody.

The monoclonal antibody can be produced by the following method.

[Preparation of Monoclonal Antibody]

(a) Preparation of Monoclonal Antibody-Producing Cells.

The ligand polypeptide is administered to warm-blooded animals either solely or together with carriers

or diluents to the site where the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once every two to six weeks and two to ten times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats and chickens and the use of mice and rats is preferred.

In the preparation of the cells which produce monoclonal antibodies, an animal wherein the antibody titer is noted is selected from warm-blooded animals (e.g. mice) immunized with antigens, then spleen or lymph node is collected after two to five days from the final immunization and antibody-producing cells contained therein are fused with myeloma cells to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may, for example, be carried out by reacting a labeled ligand polypeptide or a labeled G protein-coupled receptor protein (which will be mentioned later) with the antiserum followed by measuring the binding activity of the labeling agent with the antibody. The operation for fusing may be carried out, for example, by the method of Koehler and Milstein (Nature, 256, 495, 1975). Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc. and the use of PEG is preferred.

Examples of the myeloma cells are NS-1, P3U1, SP2/0, AP-1, etc. and the use of P3U1 is preferred. The preferred fusion ratio of the numbers of antibody-producing cells used (spleen cells) to the numbers of myeloma cells is within a range of about 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of about 10-80% followed by incubating at 20-40°C (preferably, at 30-37°C) for one to ten

minutes, an efficient cell fusion can be carried out.

Various methods may be applied for screening a hybridoma which produces anti-ligand polypeptide antibody. For example, a supernatant liquid of hybridoma culture is added to a solid phase (e.g. microplate) to which the ligand polypeptide antigen is adsorbed either directly or with a carrier, then anti-immunoglobulin antibody (anti-mouse immunoglobulin antibody is used when the cells used for the cell fusion are those of mouse) which is labeled with a radioactive substance, an enzyme or the like, or protein A is added thereto and then anti-ligand polypeptide monoclonal antibodies bound on the solid phase are detected; or a supernatant liquid of the hybridoma culture is added to the solid phase to which anti-immunoglobulin or protein A is adsorbed, then the ligand polypeptide labeled with a radioactive substance or an enzyme is added and anti-ligand polypeptide monoclonal antibodies bonded with the solid phase is detected.

Selection and cloning of the anti-ligand polypeptide monoclonal antibody-producing hybridoma may be carried out by methods per se known to those of skill in the art or methods similar thereto. Usually, it is carried out in a medium for animal cells, containing HAT (hypoxanthine, aminopterin and thymidine). With respect to a medium for the selection, for the cloning and for the growth, any medium may be used so far as hybridoma is able to grow therein. Examples of the medium are an RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Japan) containing 1-20% (preferably 10-20%) of fetal calf serum (FCS), a GIT medium (Wako Pure Chemical, Japan) containing 1-20% of fetal calf serum and a serum-free medium for hybridoma culturing (SFM-101; Nissui Seiyaku, Japan). The culturing temperature is usually

20-40°C and, preferably, about 37°C. The culturing time is usually from five days to three weeks and, preferably, one to two weeks. The culturing is usually carried out in 5% carbon dioxide gas. The antibody titer of the supernatant liquid of the hybridoma culture may be measured in the same manner as the above-mentioned measurement of the antibody titer of the anti-ligand polypeptide in the antiserum.

(b) Purification of the Monoclonal Antibody.

In the same manner as the separation/purification of conventional polyclonal antibodies, the separation/purification of the anti-ligand polypeptide monoclonal antibody may be carried out by methods for separating/purifying immunoglobulin such as salting-out, precipitation with an alcohol, isoelectric precipitation, electrophoresis, adsorption/deadsorption using ion exchangers such as DEAE, ultracentrifugation, gel filtration, specific purifying methods in which only an antibody is collected by treatment with an active adsorbent such as an antigen-binding solid phase, protein A or protein G and the bond is dissociated whereupon the antibody is obtained.

The ligand polypeptide antibody which is manufactured by the aforementioned method (a) or (b) is capable of specifically recognizing ligand polypeptide, accordingly, it can be used for a quantitative determination of the ligand polypeptide in test liquid samples and particularly for a quantitative determination by sandwich immunoassays.

Thus, the present invention provides, for example, the following methods:

(i) a quantitative determination of a ligand polypeptide in a test liquid sample, which comprises (a) competitively reacting the test liquid sample and a labeled ligand polypeptide with an antibody which reacts with the ligand polypeptide, and

(b) measuring the ratio of the labeled ligand polypeptide binding with said antibody; and
(ii) a quantitative determination of a ligand polypeptide in a test liquid sample, which comprises (a) reacting the test liquid sample with an antibody immobilized on an insoluble carrier and a labeled antibody simultaneously or continuously, and
(b) measuring the activity of the labeling agent on the insoluble carrier
wherein one antibody is capable of recognizing the N-terminal region of the ligand polypeptide while another antibody is capable of recognizing the C-terminal region of the ligand polypeptide.

When the monoclonal antibody of the present invention recognizing a ligand polypeptide (hereinafter, may be referred to as "anti-ligand polypeptide antibody") is used, ligand polypeptide can be measued and, moreover, can be detected by means of a tissue staining, etc. as well. For such an object, antibody molecules per se may be used or $F(ab')_2$, Fab' or Fab fractions of the antibody molecule may be used too. There is no particular limitation for the measuring method using the antibody of the present invention and any measuring method may be used so far as it relates to a method in which the amount of antibody, antigen or antibody-antigen complex, depending on or corresponding to the amount of antigen, e.g. the amount of ligand polypeptide, etc. in the liquid sample to be measured, is detected by a chemical or a physical means and then calculated using a standard curve prepared by a standard solution containing the known amount of antigen. For exmaple, nephrometry, competitive method, immunometric method and sandwich method are suitably used and, in terms of sensitivity and specificity, the sandwich method which will be described herein later is particularly

preferred.

Examples of the labeling agent used in the measuring method using the labeling substance are radioisotopes, enzymes, fluorescent substances, luminescent substances, colloids, magnetic substances, etc. Examples of the radioisotope are [^{125}I], [^{131}I], [^{3}H] and [^{14}C]; preferred examples of the enzyme are those which are stable and with big specific activity, such as β-galactosidase, β-glucosidase, alkali phosphatase, peroxidase and malate dehydrogenase; examples of the fluorescent substance are fluorescamine, fluorescein isothiocyanate, etc.; and examples of the luminescent substance are luminol, luminol derivatives, luciferin, lucigenin, etc. Further, a biotin-avidin system may also be used for binding an antibody or antigen with a labeling agent.

In an insolubilization (immobilization) of antigens or antibodies, a physical adsorption may be used or a chemical binding which is usually used for insolubilization or immobilization of proteins or enzymes may be used as well. Examples of the carrier are insoluble polysaccharides such as agarose, dextran and cellulose; synthetic resins such as polystyrene, polyacrylamide and silicone; glass; etc.

In a sandwich (or two-site) method, the test liquid is subjected to reaction with an insolubilized anti-ligand polypeptide antibody (the first reaction), then it is subjected to reaction with a labeled anti-ligand polypeptide antibody (the second reaction) and the activity of the labeling agent on the insoluble carrier is measured whereupon the amount of the ligand polypeptide in the test liquid can be determined. The first reaction and the second reaction may be conducted reversely or simultaneously or they may be conducted with an interval. The type of the labeling agent and the method of insolubilization (immobilization) may be

the same as those mentioned already herein. In the immunoassay by means of a sandwich method, it is not always necessary that the antibody used for the labeled antibody and the antibody for the solid phase is one type or one species but, with an object of improving the measuring sensitivity, etc., a mixture of two or more antibodies may be used too.

In the method of measuring ligand polypeptide by the sandwich method of the present invention, the preferred anti-ligand polypeptide antibodies used for the first and the second reactions are antibodies wherein their sites binding to the ligand polypeptide are different each other. Thus, the antibodies used in the first and the second reactions are those wherein, when the antibody used in the second reaction recognizes the C-terminal region of the ligand polypeptide, then the antibody recognizing the site other than C-terminal regions, e.g. recognizing the N-terminal region, is preferably used in the first reaction.

The anti-ligand polypeptide antibody of the present invention may be used in a measuring system other than the sandwich method such as a competitive method, an immunometric method and a naphrometry. In a competitive method, an antigen in the test solution and a labeled antigen are subjected to reaction with an antibody in a competitive manner, then an unreacted labeled antigen (F) and a labeled antigen binding with an antibody (B) are separated (i.e. B/F separation) and the labeled amount of any of B and F is measured whereupon the amount of the antigen in the test solution is determined. With respect to a method for such a reaction, there are a liquid phase method in which a soluble antibody is used as the antibody and the B/F separation is conducted by polyethylene glycol, a second antibody to the above-mentioned antibody,

etc.; and a solid phase method in which an immobilized antibody is used as the first antibody or a soluble antibody is used as the first antibody while an immobilized antibody is used as the second antibody.

In an immunometric method, an antigen in the test solution and an immobilized antigen are subjected to a competitive reaction with a certain amount of a labeled antibody followed by separating into solid and liquid phases; or the antigen in the test solution and an excess amount of labeled antibody are reacted, then a immobilized antigen is added to bind an unreacted labeled antibody with the solid phase and separated into solid and liquid phases. After that, the labeled amount of any of the phases is measured to determine the antigen amount in the test solution.

In a nephrometry, the amount of insoluble sediment which is produced as a result of the antigen-antibody reaction in a gel or in a solution is measured. Even when the antigen amount in the test solution is small and only a small amount of the sediment is obtained, a laser nephrometry wherein scattering of laser is utilized can be suitably used.

In applying each of those immunological measuring methods (immunoassays) to the measuring method of the present invention, it is not necessary to set up any special condition, operation, etc. therefor. A measuring system (assay system) for ligand polypeptide may be constructed taking the technical consideration of the persons skilled in the art into consideration in the conventional conditions and operations for each of the methods. With details of those conventional technical means, a variety of reviews, reference books, etc. may be referred to. They are, for example, Hiroshi Irie (ed): "Radioimmunoassay" (Kodansha, Japan, 1974); Hiroshi Irie (ed): "Radioimmunoassay; Second Series" (Kodansha, Japan, 1979); Eiji Ishikawa et al.

(ed): "Enzyme Immunoassay" (Igaku Shoin, Japan, 1978); Eiji Ishikawa et al. (ed): "Enzyme Immunoassay" (Second Edition) (Igaku Shoin, Japan, 1982); Eiji Ishikawa et al. (ed): "Enzyme Immunoassay" (Third Edition) (Igaku Shoin, Japan, 1987); "Methods in Enzymology" Vol. 70 (Immunochemical Techniques (Part A)); ibid. Vo. 73 (Immunochemical Techniques (Part B)); ibid. Vo. 74 (Immunochemical Techniques (Part C)); ibid. Vo. 84 (Immunochemical Techniques (Part D: Selected Immunoassays)); ibid. Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods)); ibid. Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies)) (Academic Press); etc.

As such, the amount of ligand polypeptide can now be determined with a high precision using the anti-ligand polypeptide antibody of the present invention.

(5) Construction of a non-human transgenic animal

The present invention further provides a non-human mammal harboring a foreign DNA coding for the ligand polypeptide of the present invention (hereinafter referred to briefly as foreign DNA) or a mutant thereof (sometimes referred to briefly as a foreign mutant DNA).

Thus, the present invention provides

(1) a non-human mammal harboring a foreign DNA of the present invention or a foreign mutant DNA thereof;

(2) the non-human mammal according to (1) which is a rodent;

(3) the non-human mammalian according to (2) wherein the rodent is a mouse or rat;

(4) a recombinant vector containing the foreign DNA of the present invention or a foreign mutant DNA thereof and capable of being expressed in a mammal; and

(5) a pharmaceutical composition for gene theraphy

which comprises the recombinant vector according to (4).

Examples of the DNA coding for the ligand polypeptide of the present invention is a DNA comprises the DNA having a nucleotide sequence encoding the polypeptide comprising the amino acid sequence represented by SEQ ID NO:1, or a substantial equivalent thereof (hereinafter, may simply referred as the DNA of the present invention), such as the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 or SEQ ID NO:3.

The non-human mammal harboring the foreign DNA of the present invention or a foreign mutant DNA thereof (hereinafter referred to briefly as the transgenic animal of the present invention) can be constructed by transferring the objective DNA to a germinal cell such as an unfertilized egg cell, fertilized egg cell, or sperm cell or its primordial cell, preferably in the period of embryogenesis in the ontogenesis of a non-human mammal (more preferably in the stage of a single cell or a fertilized egg cell and generally at the 8-cell stage or earlier), by the calcium phosphate method, electric pulse method, lipofection method, agglutination method, microinjection method, particle gun method, or DEAE-dextran method.

Further, by using above-mentioned transferring method, the DNA of the present invention can be introduced into somatic cells, various organs in the body, tissue cells and can be used for cell cultivation, tissue cultivation, etc. Moreover, these cells can be hybridized with above-mentioned germinal cell to produce the non-human transgenic mammal.

The non-human mammal used includes bovine, swine, sheep, goat, rabbit, canine, feline, guinea pig, hamster, murine, rat, and so on. From the standpoint of construction of a diseased animal model, rodents

which have comparatively short ontogenesis and life cycles and can be easily bred, particularly mice (e.g. pure strains such as C57BL/6, DBA2, etc. and hybrid strains such as $B6C3F_1$, BDF_1, $B6D2F_1$, BALB/c, ICR, etc.) or rats (e.g. Wistar, SD, etc.) are preferred.

The "mammal" as mentioned with reference to the recombinant vector capable of being expressed in a mammal includes the same non-human mammals as those mentioned above and humans.

The foreign DNA of the present invention may be one derived from a mammal of the same species as the host animal or a mammal of a different species. For transfer of the DNA of the present invention to the host animal, it is generally advantageous to use a DNA construct prepared by linking the DNA at downstream of a promoter capable of being expressed in animal cells. For example, in transferring the human-derived DNA of the present invention, this human DNA of the present invention can be linked at downstream of a promoter capable of causing expression of DNAs derived from various animals (e.g. rabbit, canine, feline, guinea pig, hamster, rat, murine, etc.) harboring the DNA of the present invention having high homology thereto to prepare a DNA construct (e.g. a vector) which can then be microinjected into a fertilized egg cell of a host mammal such as a fertilized murine egg cell, whereby a transgenic mammal showing a high expression of the DNA of the present invention can be provided.

Examples of the expression vector used for the protein of the present invention are plasmids derived from E. coli, plasmids derived from B. subtilis, plasmids of the yeast origin, λ phage and other bacteriophages, retroviruses such as Molony leukemia virus, and animal viruses such as vaccinia virus and vaculovirus. Preferable examples are plasmids of the E. coli origin, plasmids of the B. subtilis origin, and

yeast-derived plasmids.

The promoter for the regulation of the expression of the DNA are (1) promoters for DNAs derived from viruses (e.g. simian virus, cytomegalovirus, Molony leukemia virus, JC virus, papilloma virus, poliovirus, etc.), (2) promoters derived from mammals (e.g. man, rabbit, dog, cat, guinea pig, hamster, rat, mouse, etc.) for albumin, insulin II, uroprakin II, elastase, erythropoietin, endothelin, muscle creatine kinase, glial fibrillary acidic protein, glutathione S-transferase, platelet-derived growth factor β, keratin K1, K10, and K14, collagen type I and type II, cyclic AMP-dependent protein kinase βI subunit, dystrophin, tartaric acid-resistant alkaline phosphatase, atrial natriuretic factor, endothelial receptor tyrosine kinase (generally abbreviated as Tie2), sodium/potassium-exchanging adenosinetriphosphatase (Na^+, K^+-ATPase), neurofilament light chain, metallothionein I and IIA, metalloprotease I tissue inhibitor, MHC Class I antigen (H-2L), H-ras, renin, dopamine β-hydroxylase, thyroid peroxidase (TPO), polypeptide chain elongation factor 1α (EF-1α), β actin, α- and β-myosin heavy chain, myosin light chains 1 and 2, myelin basic protein, thyroglobulin, Thy-1, immunoglobulin H chain variable region (VNP), serum amyloid P component, myoglobin, troponin C, smooth muscle α-actin, preproenkephalin A or vasopressin, and so on. Preferable promoters are promoters conducive to high expression in the whole body, such as cytomegalovirus promoter, human polypeptide chain elongation factor 1α (EF-1α) promoter, and human and chicken β-actin promoters.

The vector preferably has a sequence for terminating the transcription of the objective mRNA (generally called terminator) in the transgenic mammal. The examples of the sequence are sequences derived from

viruses, various mammals. Preferable examples are the SV40 terminator derived from simian virus, and so on.

In addition, for enhancing the expression of the objective DNA, it is possible, depending on the specific objective, to link the splicing signal, enhancer domain, a portion of the eucaryotic DNA intron, etc. at upstream of the 5'-end of the promoter region, between the promoter region and the translated region, or at downstream of the 3'-end of the translated region.

The translation region of the normal protein of the present invention can be obtained, as the whole or part of the genomic DNA, from the DNAs derived from the liver, kidney, or thyroid cells or fibroblasts of various mammals (e.g. rabbit, canine, feline, guinea pig, hamster, rat, murine, man, etc.) or from various commercial genomic DNA libraries, or starting with the complementary DNAs prepared from RNAs derived from the liver, kidney, thyroid cells or fibroblasts by the known technique. The foreign abnormal DNA can be constructed by mutating the translated region of the normal protein obtained from the above-mentioned cells or tissues by the mutagenesis method.

The translated region can be prepared as a DNA construct which can be expressed in a transgenic animal, by the routine recombinant DNA technique, i.e. by coupling it at downstream of the promoter and, if desired, at upstream of the transcription termination site.

The transfer of the DNA of the present invention at the fertilized egg cell stage insures that the DNA will be ubiquitous in all the germ cells and somatic cells of the host mammal. The presence of the DNA of the present invention in the germ cells of the transgenic animal following DNA transfer means that all the germ cells and somatic cells of all the progeny of

the transgenic animal harbor the DNA of the present invention. Thus, the offspring of animals of this line to which DNA is passed down have the DNA of the present invention in their germ cells and somatic cells.

The non-human mammal to which the foreign normal DNA of the present invention has been transferred can be verified by mating to retain the DNA stably and then bred as a strain harboring the transferred DNA from generation to generation under the usual breeding conditions. The transfer of the DNA of the present invention in the fertilized egg cell stage is carried out in such a manner that the transferred DNA will be present in excess in all the germ cells and somatic cells of the transgenic animal. The presence of an excess of the DNA of the present invention in the germ cells of the transgenic animal means that all the progeny of this line harbor an excess of the DNA of the present invention in their germ cells and somatic cells. By preparing homozygous animals having the transferred DNA in both homologous chromosomes and mating the animals of both sexes, they can be bred serially so that all the progeny may harbor an excess of the DNA.

The non-human mammal harboring the normal DNA of the present invention features a high expression of the DNA and may eventually develop a hyperergasia of the protein of the present invention through activation of the function of the endogenous normal DNA and, therefore, can be utilized as an animal model of the disease. For example, by using the transgenic animal harboring the normal DNA of the present invention, it is possible to study the hyperergasia of the protein of the present invention to elucidate the mechanisms of diseases with which the protein of the present invention is associated, and explore therapeutic modalities for the diseases.

Furthermore, the mammal to which the foreign normal DNA of the present invention has been transferred develops symptoms due to an increase in the free protein of the present invention and, therefore, can also be used in the screening of therapeutic drugs for diseases with which the protein of the present invention is associated.

On the other hand, the non-human mammal harboring the foreign abnormal DNA of the present invention can be verified by mating to retain the DNA stably and then bred as a line harboring the DNA from generation to generation under the usual breeding conditions. Moreover, it is possible to incorporate the objective DNA in the above-mentioned plasmid for use as a starting material. The DNA construct with the promoter can be prepared by the routine recombinant DNA technique. Transfer of the abnormal DNA of the present invention in the fertilized egg cell stage insures that the transferred DNA will be ubiquitous in all the germ cells and somatic cells of the host mammal. The presence of the abnormal DNA of the present invention in the germ cells of the transgenic animal means that all the offspring of this transgenic animal harbor the abnormal DNA of the present invention in all of their germ cells and somatic cells. The progeny of this animal harbor the abnormal DNA of the present invention in all of their germ cells and somatic cells. By preparing homozygous male and female animals having the introduced DNA in both homologous chromosomes and mating them, it can be insured that all their offsprings harbor the DNA.

The non-human mammal harboring the abnormal DNA of the present invention features a high expression of the abnormal DNA and, therefore, may eventually develop adiaphoria associated with functional inactivation of the protein of the present invention through inhibition

of the function of the endogenous normal DNA and, therefore, can be utilized as an animal model of the disease. For example, by using the transgenic animal harboring the abnormal DNA of the present invention, analysis of the mechanism of this functional inactivation adiaphoria due to the protein of the present invention and therapeutic modalities for the disease can be explored.

As a specific potential use, the transgenic animal with a high expression of the abnormal DNA of the present invention can be used as a model for elucidating the functional inhibition of the normal protein by the abnormal protein of the present invention (dominant negative effect) in adiaphoria of functional inactivation type due to the protein of the present invention. Moreover, the transgenic mammal harboring the foreign abnormal DNA of the present invention develops symptoms due to an decrease in the protein of the present invention and, therefore, can be utilized in the screening of therapeutic compounds for adiaphoria due to functional inactivation of the protein of the present invention.

As other potential uses for transgenic animals harboring the two kinds of DNAs described above, the following uses can be suggested.

(1) Use as a cell source for tissue culture;

(2) Analysis of the relationship of the protein of the present invention to proteins which are specifically expressed or activated by the protein by direct analysis of DNAs or RNAs in the tissues of the transgenic mammal harboring the DNA of the present invention or analysis of the composition of the protein expressed by the DNA;

(3) Study of the functions of cells of those tissues which are generally difficult to culture by using the cells from the tissues containing the DNA as cultured

by the standard tissue culture technique;

(4) Screening of drugs capable of enhancing the cell functions by using the cells described in (3);

(5) Isolation and purification of the muteins of the present invention and construction of antibodies to the muteins.

Furthermore, by using the transgenic animal of the present invention, clinical symptoms of diseases associated with the protein of the present invention, inclusive of said adiaphoria associated with functional inactivation of the protein of the present invention, can be investigated. In addition, more detailed pathological findings can be generated in various organs of this model of diseases associated with the protein of the present invention, thus contributing to the development of new therapies and the study and treatment of secondary diseases arising from such diseases.

Moreover, following isolation of various organs from the transgenic animal of the present invention and their mincing and digestion with a proteolytic enzyme such as trypsin, free single cells harboring the transferred gene can be recovered and cultured for establishment of a cell line. Furthermore, characterization of cells producing the protein of the present invention can be made and their relationship to apotosis, differentiation, or proliferation, the mechanism of signal transduction in them, and abnormalities involved can be explored to thereby generate information useful for a further elucidation of the protein of the present invention and its actions.

Moreover, for the development of therapeutic drugs for diseases associated with the protein of the present invention, such as adiaphoria due to functional inactivation of the protein of the present invention by

using the transgenic animal of the present invention, an effective and rapid screening technology for such therapeutic drugs can be established by using the test and assay methods described hereinbefore. In addition, by using the above transgenic animal or the foreign DNA expression vector of the present invention, gene therapies for diseases associated with the protein of the present invention can be explored and developed.

(6) Construction of knockout animals

The present invention further provides a non-human mammalian embryonic stem cell wherein the DNA of the present invention is inactivated and a non-human mammal deficient in expression of the DNA of the present invention wherein the DNA is inactivated.

The present invention, therefore, provides:

(1) a non-human mammalian embryonic stem cell wherein the DNA of the present invention is inactivated;

(2) the non-human mammalian embryonic stem cell according to in (1) wherein the DNA is inactivated by introduction of a reporter gene (e.g. a β-galactosidase gene of the E. coli origin);

(3) the non-human mammalian embryonic stem cell according to (1) which is neomycin-resistant;

(4) the non-human mammalian embryonic stem cell according to (1) wherein the non-human mammal is a rodent;

(5) the non-human mammalian embryonic stem cell according to (4) wherein the rodent is a mouse;

(6) a non-human mammal deficient in expression of the DNA of the present invention, wherein the DNA is inactivated;

(7) the non-human mammal according to (6) wherein the DNA is inactivated by introduction of a reporter gene (e.g. a β-galactosidase gene of E. coli origin) and the reporter gene can be expressed under the control of the

promoter against the DNA of the present invention;

(8) the non-human mammal according to (6) wherein the non-human mammal is a rodent;

(9) the non-human mammal according to (8) wherein the rodent is a mouse; and

(10) a method for screening for a compound or a salt thereof which enhances or inhibits an activity of the promoter against the DNA of the present invention, which comprises administering a test compound to the non-human mammal according to (7) and detecting an expression of the reporter gene.

The term "non-human mammalian embryonic stem cell wherein the DNA of the present invention is inactivated" means the embryonic stem cell (hereinafter referred to briefly as ES cell) of a non-human mammal in which the DNA has been deprived of the capacity to express the protein of the present invention (hereinafter referred to sometimes as the knockout DNA of the present invention) through introduction of an artificial mutation to the DNA of the present invention possessed by the non-human mammal to thereby inhibit expression of the DNA of the present invention or through substantial deprivation of the activity of the protein of the present invention which is encoded by the DNA.

The non-human mammal includes the same animals mentioned hereinbefore.

Examples of the method for introducing an artificial mutation to the DNA of the present invention are a deletion of some or all of the DNA sequence, or an insertion or substitution of a different DNA by the genetic engineering technology. By such a mutation, the codon reading frame can be shifted or the function of the promoter or exon can be disrupted to provide the knockout DNA of the present invention.

The non-human mammalian embryonic stem cell

wherein the DNA of the present invention is inactivated (hereinafter referred to as the ES cell wherein the DNA is an inactivated DNA of the DNA of the present invention or the knockout ES cell of the present invention) can be prepared by, for example, a procedure which comprises isolating the DNA of the present invention from an objective non-human mammal, inserting a drug-resistance gene, typically the neomycin-resistance gene or hygromycin-resistance gene, or a reporter gene such as lacZ (β-galactosidase gene) or cat (chloramphenicol acetyltransferase gene) in its exon region to disrupt the function of the exon or inserting a DNA sequence for terminating gene transcription (e.g. poly A coupling signal) in the intron region between exons to thereby inhibit synthesis of a complete mRNA, introducing the thus-constructed DNA chain having a DNA sequence adapted to eventually disrupt the gene (hereinafter referred to briefly as the targeting vector) into the chromosomes of the host animal by homologous recombination, subjecting the resulting ES cell to Southern hybridization analysis using the DNA sequence of the DNA of the present invention or in its vicinity as the probe or a PCR procedure using the DNA sequence of the targeting vector and a DNA sequence in the vicinity but not including the DNA of the present invention used in the construction of the targeting vector as primers, and selecting the knockout ES cell of the present invention.

Moreover, the original ES cell used for inactivation of the DNA of the present invention by the homologous recombination technique or the like may be an already established cell line such as those mentioned hereinbefore or a new cell line established de novo by the known method of Evans and Kaufma. Taking murine ES cells as an example, ES cells of the

129 line are generally employed but the immunological background of this line is not clear. Therefore, the cell line established by using BDF_1 mice created by the hybridization of C57BL/6 mice and C57BL/6 mice, both yielding few eggs, with DBA/2 mice (BDF_1 = F_1 of C57BL/6 and DBA/2) for preparing pure-line ES cells with an immunologically defined genetic background can be used with advantage. In addition to the advantage of high egg output and sturdiness of the egg, BDF_1 mice have the background of C57BL/6 mice so that in the construction of a disease model with ES cells obtained, the genetic background of the model mice can be converted to that of C57BL/6 mice by back-crossing with C57BL/6.

Moreover, in establishing an ES cell line, it is common practice to use blastocytes 3.5 days following fertilization but, aside from them, a large number of early embryos can be prepared with high efficiency by harvesting the embryos at the 8-cell stage and culturing them into blastocytes.

Furthermore, while ES cells from both male and female animals can be employed, generally ES cells of a male animal are more convenient for the construction of reproduction line chimeras. Moreover, for the purpose of reducing the burden of the complicated cultural procedure, it is preferable to carry out sexing as early as possible.

As a typical method for sexing ES cells, there can be mentioned the method in which the gene in the sex determination region on the Y chromosome is amplified and detected by PCR. Whereas the conventional karyotype analysis requires about 10^6 cells, the above method requires only about one colony equivalent of ES cells (about 50 cells). Therefore, the primary selection of ES cells in an early stage can be made by this sexing method. Since male cells can thus be

selected in the early stage, the trouble in the initial stage of culture can be drastically reduced.

Moreover, the secondary selection can be carried out by G-banding for the number of chromosomes. The number of chromosomes in the resulting ES cell is preferably 100% of the normal number but this goal may not be reached due to the physical and other factors involved in the establishment of the line. In such cases, it is preferable to knockout the gene of the ES cell and reclone it in the normal cell (taking a mouse as an example, the cell in which the number of chromosomes is 2n=40).

The embryonic stem cell line thus established is generally very satisfactory in proliferation characteristic but since it is liable to lose its ontogenic ability, it must be subcultured with sufficient care. For example, this cell line should be cultured on suitable feeder cells such as STO fibroblasts in the presence of LIF (1-10000 U/ml) in a carbon dioxide incubator (preferably 5% CO_2-95% air or 5% oxygen-5% CO_2-90% air) at about 37°C and, in subculture, it should be treated with trypsin/EDTA solution (generally 0.001-0.5% trypsin/0.1-5 mM EDTA, preferably about 0.1% trypsin/1 mM EDTA) to provide single cells and seed them on freshly prepared feeder cells. While such subculture is generally performed every 1-3 days, it is good practice to observe the cells on each occasion and, whenever morphologically abnormal cells are discovered, discard the culture.

ES cells can be allowed to differentiate into various types of cells, such as head long muscle cells, visceral muscle cells, heart muscle cells, etc. by conducting monolayer culture to a high density under suitable conditions or suspension culture until a mass of cells is formed (M. J. Evans & M. H. Kaufman, Nature, 292, 154, 1981; G. R. Martin, Proceedings of

National Academy of Science USA, 78, 7634, 1981; T. C. Doetschman et al., Journal of Embryology and Experimental Morphology, 87, 27, 1985), and the cell deficient in expression of the DNA of the present invention as obtained by causing the ES cell of the present invention to differentiate is useful for the cytobiological in vitro study of the polypeptide of the present invention.

The non-human mammal deficient in expression of the DNA of the present invention can be differentiated from the normal animal by assaying the mRNA in the animals by the known method and comparing the amounts of expression indirectly.

The non-human mammal used for this purpose includes the animals mentioned hereinbefore.

Referring to the non-human mammal deficient in expression of the DNA of the present invention, the DNA of the present invention can be knocked out by introducing the targeting vector constructed as above into, for example, a murine embryonic stem cell or a murine egg cell and thereby causing the DNA sequence of the targeting vector harboring the inactivated DNA of the present invention to undergo homologous recombination with, and accordingly replacing, the DNA of the present invention on the murine embryonic stem cell or egg cell chromosomes.

The cell with the DNA of the present invention thus knocked out can be obtained by Southern hybridization analysis using a DNA sequence of the DNA of the present invention or in its vicinity as a probe or by PCR using a DNA sequence of the targeting vector or a murine-derived DNA sequence in a region adjacent to but not including the DNA of the present invention used in the targeting vector as primers. When a non-human mammalian embryonic stem cell is used, a cell line with the DNA of the present invention knocked out

by the homologous recombination technique is cloned and injected into the non-human mammalian embryo or blastocyte at a suitable stage of embryogenesis, for example at the 8-cell stage, and the resulting chimera embryo is transplanted in the pseudopregnant uterus of the non-human mammal. The animal thus obtained is a chimera animal constituted by both the cells harboring the normal DNA of the present invention and the cells harboring the artificially mutated DNA of the present invention.

When some of the gametes of this chimera animal harbor the mutated DNA of the present invention, an individual of which the entire tissues are constituted by cells harboring the mutated DNA of the present invention can be screened from the colony of animals obtained by crossing such a chimera animal with a normal animal, for example by coat color discrimination. The individuals thus selected are usually animals deficient in hetero-expression of the protein of the present invention and by mating such individuals deficient in hetero-expression of the protein of the present invention with each other, animals deficient in homo-expression of the protein of the present invention can be acquired.

When an egg cell is used, a transgenic non-human mammal with the targeting vector having been introduced into its chromosomes can be prepared by injecting the DNA solution into the egg cell nucleus by the microinjection technique and selecting animals expressing a mutation of the DNA of the present invention by homologous recombination.

The individuals with the DNA of the present invention knocked out are mated to verify that the animals obtained by mating also have the DNA knocked out and they can be sub-bred under the usual breeding conditions.

Preparation and maintenance of the reproduction line can also be carried out in the routine manner. Thus, by mating male and female animals harboring the inactivated DNA, homozygotes having the inactivated DNA in both homologous chromosomes can be obtained. The homozygotes thus obtained are bred under such conditions that, with regard to the dam, the number of homozygotes is plural per normal individual. By mating male and female heterozygotes, homozygotes and heterozygotes both harboring the inactivated DNA can be sub-bread.

The non-human mammalian embryonic stem cell harboring the inactivated DNA of the present invention is very useful for the construction of non-human mammals deficient in expression of the DNA of the present invention. Moreover, the mouse deficient in expression of the protein of the present invention lacks the various biological activities inducible by the protein of the present invention and can, therefore, be of use as an animal model of diseases arising from inactivation of the biological activities of the protein of the present invention, thus being of use in the etiological studies of diseases and development of therapeutics.

In non-human mammals deficient in expression of the DNA of the present invention wherein the DNA of the present invention is inactivated by introducing a reporter gene, the reporter gene is under the control of the promoter for the DNA of the present invention and, therefore, the activity of the promoter can be detected by tracing the expression of the substance encoded by the reporter gene.

For instance, when part of the DNA region coding for the protein of the present present invention has been inactivated by Escherichia coli-derived β-galactosidase gene (lacZ), β-galactosidase is expressed

in those tissues in which, the protein of the present invention would have been expressed. Therefore, the status of expression of the protein of the present invention in a living animal body can be traced, easily and expediently, for example, by the staining method using a reagent serving as a substrate for β-galactosidase, such as 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal). More specifically, a tissue section of a mouse defective in the protein of the present invention is fixed with glutaraldehyde or the like, washed with Dulbecco's phosphate-buffered saline (PBS), and reacted with a staining solution containing X-gal at room temperature or around 37°C for about 30 minutes to 1 hour. The tissue sample is then washed with 1 mM EDTA/PBS solution to terminate the β-galactosidase reaction and observed for color development. Alternatively, the mRNA coding for lacZ may be detected by a conventional method.

The non-human mammals deficient in expression of the DNA of the present invention is very useful for screening the compounds which activate or inactivate the promoter of the polypeptide of the present invention, and can contribute to find the mechanism of the diseases derived from the deficiency of producing the polypeptide of the present invention or to develop the drug for treating such diseases.

In the specification and drawings of the present application, the abbreviations used for bases (nucleotides), amino acids and so forth are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or those conventionally used in the art. Examples thereof are given below. Amino acids for which optical isomerism is possible are, unless otherwise specified, in the L form.

DNA : Deoxyribonucleic acid

cDNA : Complementary deoxyribonucleic acid

A : Adenine
T : Thymine
G : Guanine
C : Cytosine
RNA : Ribonucleic acid
mRNA : Messenger ribonucleic acid
dATP : Deoxyadenosine triphosphate
dTTP : Deoxythymidine triphosphate
dGTP : Deoxyguanosine triphosphate
dCTP : Deoxycytidine triphosphate
ATP : Adenosine triphosphate
EDTA : Ethylenediamine tetraacetic acid
SDS : Sodium dodecyl sulfate
EIA : Enzyme Immunoassay
G, Gly: Glycine (or Glycyl)
A, Ala: Alanine (or Alanyl)
V, Val: Valine (or Valyl)
L, Leu: Leucine (or Leucyl)
I, Ile: Isoleucine (or Isoleucyl)
S, Ser: Serine (or Seryl)
T, Thr: Threonine (or Threonyl)
C, Cys: Cysteine (or Cysteinyl)
M, Met: Methionine (or Methionyl)
E, Glu: Glutamic acid (or Glutamyl)
D, Asp: Aspartic acid (or Aspartyl)
K, Lys: Lysine (or Lysyl)
R, Arg: Arginine (or Arginyl)
H, His: Histidine (or Histidyl)
F, Phe: Phenylalamine (or Phenylalanyl)
Y, Tyr: Tyrossine (or Tyrosyl)
W, Trp: Tryptophan (or Tryptophanyl)
P, Pro: Proline (or Prolyl)
N, Asn: Asparagine (or Asparaginyl)
Q, Gln: Glutamine (or Glutaminyl)
pGlu: Pyroglutamic acid (or Pyroglutamyl)
Me: Methyl

Et: Ethyl
Bu: Butyl
Ph: Phenyl
TC: Thiazolidinyl-4(R)-carboxamide

In this specification, substitutions, protective groups and reagents commonly used are indicated by the following abbreviations:

BHA : benzhydrylamine
pMBHA : p-methylbenzhydrylamine
Tos : p-toluenesulfonyl
CHO : formyl
HONB : N-hydroxy-5-norbornene-2,3-dicarboxyimide
OcHex : cyclohexyl ester
Bzl : benzyl
Bom : benzyloxymethyl
Br-Z : 2-bromobenzyloxycarbonyl
Boc : t-butoxycarbonyl
DCM : dichloromethane
HOBt : 1-hydroxybenztriazole
DCC : N,N'-dicyclohexylcarbodiimide
TFA : trifluoro acetate
DIEA : diisopropylethylamine
Fmoc : N-9-fluorenylmethoxycarbonyl
DNP : dinitrophenyl
Bum : t-butoxymethyl
Trt : trityl

Each SEQ ID NO set forth in the SEQUENCE LISTING of the specification refers to the following sequence:
[SEQ ID NO:1] is an entire amino acid sequence of the murine pituitary-derived ligand polypeptide encoded by the cDNA included in pBOV3.
[SEQ ID NO:2] is an entire nucleotide sequence of the murine pituitary-derived ligand polypeptide cDNA.
[SEQ ID NO:3] is a genomic nucleotide sequence of the murine pituitary-derived ligand polypeptide cDNA.
[SEQ ID NO:4] is an entire amino acid sequence of the

matured murine pituitary-derived ligand polypeptide encoded by the cDNA included in pBOV3.

[SEQ ID NO:5] is an amino acid sequence of the antigen which can be used for preparation of the anti-ligand polypeptide antibody.

[SEQ ID NO:6] is an amino acid sequence of the antigen which can be used for preparation of the anti-ligand polypeptide antibody.

[SEQ ID NO:7] is an amino acid sequence of the antigen which can be used for preparation of the anti-ligand polypeptide antibody.

[SEQ ID NO:8] is an amino acid sequence of the antigen which can be used for preparation of the anti-ligand polypeptide antibody.

[SEQ ID NO:9] is an amino acid sequence of the antigen which can be used for preparation of the anti-ligand polypeptide antibody.

[SEQ ID NO:10] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment included in p19P2.

[SEQ ID NO:11] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:12] is an entire amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA include in phGR3.

[SEQ ID NO:13] is a partial amino acid sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein encoded by the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragment having a nucleotide

sequence (SEQ ID NO:18), derived based upon the nucleotide sequences of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO:14] is a partial amino acid sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein encoded by p5S38.

[SEQ ID NO:15] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:16] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:17] is an entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNa include in phGR3.

[SEQ ID NO:18] is a nucleotide sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA, derived based upon the nucleotide sequences of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO:19] is a nucleotide sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA include in p5S38.

[SEQ ID NO:20] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:21] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:22] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:23] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor

protein.

[SEQ ID NO:24] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:25] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:26] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-3 fraction. The amino acid sequence corresponds to 23rd to 51st positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:27] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-2 fraction. The amino acid sequence corresponds to 34th to 52nd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:28] is entire amino acid sequence of the murine pituitary-derived ligand polypeptide encoded by the cDNA included in pBOV3.

[SEQ ID NO:29] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P5-1.

[SEQ ID NO:30] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-1.

[SEQ ID NO:31] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-2.

[SEQ ID NO:32] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PE.

[SEQ ID NO:33] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand

polypeptide, wherein the primer is represented by PDN.
[SEQ ID NO:34] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FB.
[SEQ ID NO:35] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FC.
[SEQ ID NO:36] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVF.
[SEQ ID NO:37] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVR.
[SEQ ID NO:38] is an entire amino acid sequence of the bovine genome-derived ligand polypeptide.
[SEQ ID NO:39] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 53rd positions of the amino acid sequence of SEQ ID NO:1.
[SEQ ID NO:40] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 54th positions of the amino acid sequence of SEQ ID NO:1.
[SEQ ID NO:41] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO:1.
[SEQ ID NO:42] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO:1.
[SEQ ID NO:43] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO:1.
[SEQ ID NO:44] is an amino acid sequence of the bovine

pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:45] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by RA.

[SEQ ID NO:46] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by RC.

[SEQ ID NO:47] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by rF.

[SEQ ID NO:48] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by rR.

[SEQ ID NO:49] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by R1.

[SEQ ID NO:50] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by R3.

[SEQ ID NO:51] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by R4.

[SEQ ID NO:52] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by HA.

[SEQ ID NO:53] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by HB.

[SEQ ID NO:54] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by HE.

[SEQ ID NO:55] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by HF.

[SEQ ID NO:56] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by 5H.
[SEQ ID NO:57] is a synthetic DNA primer for screening of cDNA coding for the human-derived ligand polypeptide, wherein the primer is represented by 3HN.
[SEQ ID NO:58] is an entire nucleotide sequence of the bovine pituitary-derived ligand polypeptide cDNA.
[SEQ ID NO:59] is an entire nucleotide sequence of the murine-derived ligand polypeptide cDNA.
[SEQ ID NO:60] is an entire nucleotide sequence of the human-derived ligand polypeptide cDNA.
[SEQ ID NO:61] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by rFBG.
[SEQ ID NO:62] is a synthetic DNA primer for screening of cDNA coding for the murine-derived ligand polypeptide, wherein the primer is represented by rRSA.

The transformant Escherichia coli, designated INVαF'/p19P2, which is obtained in the Example 2 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with the National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan and has been assigned the Accession Number FERM BP-4776. It is also on deposit from August 22, 1994 with the Institute for Fermentation, Osaka, Japan (IFO) and has been assigned the Accession Number IFO 15739.

The transformant Escherichia coli, designated INVαF'/pG3-2, which is obtained in the Example 4 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with NIBH and has been assigned the Accession Number FERM BP-4775. It is also on deposit from August 22, 1994 with IFO and has been assigned the Accession Number IFO

15740.

The transformant Escherichia coli, designated JM109/phGR3, which is obtained in the Example 5 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4807. It is also on deposit from September 22, 1994 with IFO and has been assigned the Accession Number IFO 15748.

The transformant Escherichia coli, designated JM109/p5S38, which is obtained in the Example 8 mentioned herein below, is on deposit under the terms of the Budapest Treaty from October 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4856. It is also on deposit from October 25, 1994 with IFO and has been assigned the Accession Number IFO 15754.

The transformant Escherichia coli, designated JM109/pBOV3, which is obtained in the Example 20 mentioned herein below, is on deposit under the terms of the Budapest Treaty from February 13, 1996, with NIBH and has been assigned the Accession Number FERM BP-5391. It is also on deposit from January 25, 1996 with IFO and has been assigned the Accession Number IFO 15910.

The transformant Escherichia coli, designated JM109/pRAV3, which is obtained in the Example 29 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5665. It is also on deposit from September 3, 1996 with IFO and has been assigned the Accession Number IFO 16012.

The transformant Escherichia coli, designated JM109/pHOV7, which is obtained in the Example 32 mentioned herein below, is on deposit under the terms

of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5666. It is also on deposit from September 5, 1996 with IFO and has been assigned the Accession Number IFO 16013.

The transformant Escherichia coli, designated JM109/pmGB3, which is obtained in the Example 33 mentioned herein below, is on deposit under the terms of the Budapest Treaty from March 3, 1997, with NIBH and has been assigned the Accession Number FERM BP-5666. It is also on deposit from February 19, 1997 with IFO and has been assigned the Accession Number IFO 16059.

[Industrial Application]

The bioactive substance of the present invention, namely the ligand polypeptide or its amide or ester thereof, or a salt thereof, a partial peptide thereof, or the DNA coding for said ligand polypeptide, has function modulating activity for various tissues or internal organs, e.g. heart, lung, liver, spleen, thymus, kidney, adrenal glands, skeltal muscle, testis etc., besides pituitary, central nervous system or pancreas, and are useful as medicines. The substance also is useful for the screening of agonists or antagonists of G protein-coupled receptor proteins. The compounds which can be obtained by such screening also have function modulating activity for above-described tissues or internal organs, and are useful as medicines. Furthermore, the substance is useful for producing a non-human transgenic animal or a non-human knockout animal for analyzing the mechanism of the gene.

[Examples]

Described below are Reference Example and Examples

of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention.

[Reference Example 1]

Preparation of Synthetic DNA Primers for Amplifying DNA Coding for G protein-coupled receptor Protein

A comparison of deoxyribonucleotide sequences coding for the known amino acid sequences corresponding to or near the first membrane-spanning domain each of human-derived TRH receptor protein (HTRHR), human-derived RANTES receptor protein (L10918, HUMRANTES), human Burkitt's lymphoma-derived unknown ligand receptor protein (X68149, HSBLR1A), human-derived somatostatin receptor protein (L14856, HUMSOMAT), rat-derived μ-opioid receptor protein (U02083, RNU02083), rat-derived κ-opioid receptor protein (U00442, U00442), human-derived neuromedin B receptor protein (M73482, HUMNMBR), human-derived muscarinic acetylcholine receptor protein (X15266, HSHM4), rat-derived adrenaline α_1B receptor protein (L08609, RATAADRE01), human-derived somatostatin 3 receptor protein (M96738, HUMSSTR3X), human-derived C_5a receptor protein (HUMC5AAR), human-derived unknown ligand receptor protein (HUMRDC1A), human-derived unknown ligand receptor protein (M84605, HUMOPIODRE) and rat-derived adrenaline α_2B receptor protein (M91466, RATA2BAR) was made. As a result, highly homologous regions or parts were found.

Further, a comparison of deoxynucleotide sequences coding for the known amino acid sequences corresponding to or near the sixth membrane-spanning domain each of mouse-derived unknown ligand receptor protein (M80481, MUSGIR), human-derived bombesin receptor protein (L08893, HUMBOMB3S), human-derived adenosine A2 receptor protein (S46950, S46950), mouse-derived

unknown ligand receptor protein (D21061, MUSGPCR), mouse-derived TRH receptor protein (S43387, S43387), rat-derived neuromedin K receptor protein (J05189, RATNEURA), rat-derived adenosine A1 receptor protein (M69045, RATA1ARA), human-derived neurokinin A receptor protein (M57414, HUMNEKAR), rat-derived adenosine A3 receptor protein (M94152, DATADENREC), human-derived somatostatin 1 receptor protein (M81829, HUMSRI1A), human-derived neurokinin 3 receptor protein (S86390, S86371S4), rat-derived unknown ligand receptor protein (X61496, RNCGPCR), human-derived somatostatin 4 receptor protein (L07061, HUMSSTR4Z) and rat-derived GnRH receptor protein (M31670, RATGNRHA) was made. As a result, highly homologous regions or parts were found.

The aforementioned abbreviations in the parentheses are identifiers (reference numbers) which are indicated when GenBank/EMBL Data Bank is retrieved by using DNASIS Gene/Protein Sequencing Data Base (CD019, Hitachi Software Engineering, Japan) and are usually called "Accession Numbers" or "Entry Names". HTRHR is, however, the sequence as disclosed in Japanese Patent Publication No. 304797/1993 (EPA 638645).

Specifically, it was planned to incorporate mixed bases relying upon the base regions that were in agreement with cDNAs coding for a large number of receptor proteins in order to enhance base agreement of sequences with as many receptor cDNAs as possible even in other regions. Based upon these sequences, the degenerate synthetic DNA having a nucleotide sequence represented by SEQ ID NO:20 or SEQ ID NO:21 which is complementary to the homologous nucleotide sequence were produced.

[Synthetic DNAs]

5'-CGTGG (G or C) C (A or C) T (G or C) (G or C)

TGGGCAAC (A, G, C or T) (C or T) CCTG-3'

(SEQ ID NO:20)

5'-GT (A, G, C or T) G (A or T) (A or G) (A or G) GGCA (A, G, C or T) CCAGCAGA (G or T) GGCAAA-3'

(SEQ ID NO:21)

The parentheses indicate the incorporation of a plurality of bases, leading to multiple oligonucleotides in the primer preparation. In other words, nucleotide resides in parentheses of the aforementioned DNAs were incorporated in the presence of a mixture of plural bases at the time of synthesis.

[Example 1]

Amplification of Receptor cDNA by PCR Using Human Pituitary Gland-Derived cDNA

By using human pituitary gland-derived cDNA (QuickClone, CLONTECH Laboratories, Inc.) as a template, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out. The composition of the reaction solution consisted of the synthetic DNA primers (SEQ: 5' primer sequence and 3' primer sequence) each in an amount of 1 μM, 1 ng of the template cDNA, 0.25 mM dNTPs, 1 μl of Taq DNA polymerase and a buffer attached to the enzyme kit, and the total amount of the reaction solution was made to be 100 μl. The cycle for amplification including 95°C for 1 min., 55°C for 1 min. and 72°C for 1 min. was repeated 30 times by using a Thermal Cycler (Perkin-Elmer Co.). Prior to adding Taq DNA polymerase, the remaining reaction solution was mixed and was heated at 95°C for 5 minutes and at 65°C for 5 minutes. The amplified products were confirmed relying upon 1.2% agarose gel electrophoresis and ethidium bromide staining.

[Example 2]

Subcloning of PCR Product into Plasmid Vector and Selection of Novel Receptor Candidate Clone via

Decoding Nucleotide Sequence of Inserted cDNA Region

The PCR products were separated by using a 0.8% low-melting temperature agarose gel, the band parts were excised from the gel with a razor blade, and were heat-melted, extracted with phenol and precipitated in ethanol to recover DNAs. According to the protocol attached to a TA Cloning Kit (Invitrogen Co.), the recovered DNAs were subcloned into a plasmid vector, pCR™II (TM represents registered trademark). The recombinant vectors were introduced into E. coli INVαF' competent cells (Invitrogen Co.) to produce transformants. Then, transformant clones having a cDNA-inserted fragment were selected in an LB agar culture medium containing ampicillin and X-gal. Only transformant clones exhibiting white color were picked with a sterilized toothpick to obtain transformant Escherichia coli INVαF'/p19P2.

The individual clones were cultured overnight in an LB culture medium containing ampicillin and treated with an automatic plasmid extracting machine (Kurabo Co., Japan) to prepare plasmid DNAs. An aliquot of the DNA thus prepared was cut by EcoRI to confirm the size of the cDNA fragment that was inserted. An aliquot of the remaining DNA was further processed with RNase, extracted with phenol/chloroform, and precipitated in ethanol so as to be condensed. Sequencing was carried out by using a DyeDeoxy terminator cycle sequencing kit (ABI Co.), and the DNAs were decoded by using a fluorescent automatic sequencer, and then the data of the nucleotide sequences obtained were read by using DNASIS (Hitachi System Engineering Co., Japan). The underlined portions of Figure 1 and Figure 2 represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequences [SEQ ID NO:15 and 16

(Here, the determined nucleotide sequence is the nucleotide sequence which the underlined portion is deleted from the sequence of Figure 1 or Figure 2 respectively)].

As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment insert in the plasmid, p19P2, possessed by the transformant Escherichia coli INVαF'/p19P2. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequences were converted into amino acid sequences [SEQ ID NO:10 and 11], and homology retrieval was carried out in view of hydrophobicity plotting [Figures 3 and 4] and at the amino acid sequence level to find homology relative to neuropeptide Y receptor proteins [Figure 5].

[Example 3]

Preparation of Poly(A)$^+$RNA Fraction from Mouse Pancreatic β-Cell Strain, MIN6 and Synthesis of cDNA

A total RNA was prepared from the mouse pancreatic β-cell strain, MIN6 (Jun-ichi Miyazaki et al., Endocrinology, Vol. 127, No. 1, p.126-132) according to the guanidine thiocyanate method (Kaplan B.B. et al., Biochem. J., 183, 181-184 (1979) and, then, poly(A)$^+$RNA fractions were prepared with a mRNA purifying kit (Pharmacia Co.). Next, to 5 μg of the poly(A)$^+$RNA fraction was added a random DNA hexamer (BRL Co.) as a primer, and the resulting mixture was subjected to reaction with mouse Moloney Leukemia virus (MMLV) reverse transcriptase (BRL Co.) in the buffer attached to the MMLV reverse transcriptase kit to synthesize complementary DNAs. The reaction product was extracted with phenol/chloroform (1:1), precipitated in ethanol, and was then dissolved in 30 μl of TE buffer (10 mM Tris-HCl at pH8.0, 1 mM EDTA at pH8.0).

[Example 4]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5 µl of cDNA prepared from the mouse pancreatic β-cell strain, MIN6 in the above Example 3, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out under the same condition as in Example 1. The resulting PCR product was subcloned into the plasmid vector, pCR™II, in the same manner as in Example 2 to obtain a plasmid, pG3-2. The E. coli INVαF' was transfected with the plasmid pG3-2 to obtain transformed Escherichia coli INVαF'/pG3-2.

By using, as a template, 5 µl of the cDNA prepared from the mouse pancreatic β-cell strain, MIN6, PCR amplification using DNA primers as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a degenerate synthetic primer represented by the following sequence:

5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T) GA (C or T) (A or C) G (G or C) TAC-3'

(SEQ ID NO:22)

wherein I is inosine; and

a degenerate synthetic primer represented by the following sequence:

5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G) (C or T) GAA-3'

(SEQ ID NO:23)

wherein I is inosine,

was carried out under the same conditions as in Example 1. The resulting PCR product was subcloned into the plasmid vector, pCR™II, in the same manner as described in Example 2 to obtain a plasmid, pG1-10.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), and the DNA was decoded with the fluorescent automatic sequencer

(ABI Co.), and then the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 6 shows a mouse pancreatic β-cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:18) and an amino acid sequence (SEQ ID NO:13) encoded by the isolated DNA based upon the nucleotide sequences of plasmids pG3-2 and pG1-10. The underlined portions of Figure 6 represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 6]. As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequence was converted into an amino acid sequence [Figure 6], and hydrophobicity plotting was carried out to confirm the presence of six hydrophobic regions [Figure 8]. Upon comparing the amino acid sequence with that of p19P2 obtained in Example 2, furthermore, a high degree of homology was found as shown in [Figure 7]. As a result, it was strongly suggested that the G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 would recognize the same ligand as the G protein-coupled receptor protein encoded by p19P2 while the animal species from which the receptor proteins encoded by pG3-2 and pG1-10 were derived was different from the species from which the receptor protein encoded by p19P2 was derived.

[Example 5]

Cloning of cDNA Comprising Whole Coding Regions for Receptor Protein from Human Pituitary Gland-Derived cDNA Library

The DNA library constructed by Clontech Co. wherein λ gt11 phage vector was used (CLONTECH

Laboratories, Inc.; CLH L1139b) was employed as a human pituitary gland-derived cDNA library. The human pituitary gland cDNA library (2 x 10^6 pfu (plaque forming units)) was mixed with E. coli Y1090⁻ treated with magnesium sulfate, and incubated at 37°C for 15 minutes followed by addition of 0.5% agarose (Pharmacia Co.) LB. The E. coli was plated onto a 1.5% agar (Wako-Junyaku Co.) LB plate (containing 50 μg/ml of ampicillin). A nitrocellulose filter was placed on the plate on which plaques were formed so that the plaque was transferred onto the filter. The filter was denatured with an alkali and then heated at 80°C for 3 hours to fix DNAs.

The filter was incubated overnight at 42°C together with the probe mentioned herein below in a buffer containing 50% formamide, 5 x SSPE (20 x SSPE (pH 7.4) is 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 25 mM EDTA), 5 X Denhardt's solution (Nippon Gene, Japan), 0.1% SDS and 100 μg/ml of salmon sperm DNA for hybridization.

The probe used was obtained by cutting the DNA fragment inserted in the plasmid, p19P2', obtained in Example 2, with EcoRI, followed by recovery and labelling by incorporation of [^{32}P]dCTP (Dupont Co.) with a random prime DNA labelling kit (Amasham Co.).

It was washed with 2 x SSC (20 x SSC is 3 M NaCl, 0.3 M sodium citrate), 0.1% SDS at 55°C for 1 hour and, then, subjected to an autoradiography at -80°C to detect hybridized plaques.

In this screening, hybridization signals were recognized in three independent plaques. Each DNA was prepared from the three clones. The DNAs digested with EcoRI were subjected to agarose gel electrophoresis and were analyzed by the southern blotting using the same probe as the one used in the screening. Hybridizing bands were identified at about 0.7kb, 0.8kb and 2.0kb, respectively. Among them, the DNA fragment

corresponding to the band at about 2.0kb (λ hGR3) was selected. The λ hGR3-derived EcoRI fragment with a hybridizable size was subcloned to the EcoRI site of the plasmid, pUC18, and E. coli JM109 was transformed with the plasmid to obtain transformant E. coli JM109/phGR3. A restriction enzyme map of the plasmid, phGR3, was prepared relying upon a restriction enzyme map deduced from the nucleotide sequence as shown in Example 2. As a result, it was learned that it carried a full-length receptor protein-encoding DNA which was predicted from the receptor protein-encoding DNA as shown in Example 2.

[Example 6]

Sequencing of Human Pituitary Gland-Derived Receptor Protein cDNA

Among the EcoRI fragments inserted in the plasmid, phGR3, obtained in the above Example 5, the nucleotide sequence from EcoRI to NheI with about 1330bp that was considered to be a receptor protein-coding region was sequenced. Concretely speaking, by utilizing restriction enzyme sites that existed in the EcoRI-NheI fragments, unnecessary parts were removed or necessary fragments were subcloned in order to prepare template plasmids for analyzing the nucleotide sequence.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), and the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and then the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 9 shows a nucleotide sequence of from just after the EcoRI site up to the NheI site encoded by phGR3. The nucleotide sequence of the human pituitary gland-derived receptor protein-encoding DNA corresponds to the nucleotide sequence (SEQ ID NO:17) of from 118th

to 1227th nucleotides [Figure 9]. An amino acid sequence of the receptor protein that is encoded by the nucleotide sequence is shown in SEQ ID NO:12.

[Example 7]

Northern Hybridization with Human Pituitary Gland-Derived Receptor Protein-Encoding phGR3

Northern blotting was carried out in order to detect the expression of phGR3-encoded human pituitary gland-derived receptor proteins obtained in Example 5 in the pituitary gland at a mRNA level. Human pituitary gland mRNA (2.5 μg, Clontech Co.) was used as a template mRNA and the same as the probe used in Example 5 was used as a probe. Nylon membrane (Pall Biodyne, U.S.A.) was used as a filter for northern blotting and migration of the mRNA and adsorption (sucking) thereof with the blotting filter was carried out according to the method as disclosed in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989.

The hybridization was carried out by incubating the above-mentioned filter and probe in a buffer containing 50% formamide, 5 x SSPE, 5 X Denhardt's solution, 0.1% SDS and 100 μg/ml of salmon sperm DNA overnight at 42°C. The filter was washed with 0.1 x SSC, 0.1% SDS at 50°C and, after drying with air, was exposed to an X-ray film (XAR5, Kodak) for three days at -80°C. The results are as shown in Figure 10 from which it is considered that the receptor gene encoded by phGR3 is expressed in the human pituitary gland.

[Example 8]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5 μl of cDNA prepared from the mouse pancreatic β-cell strain, MIN6 in Example 3, PCR amplification using the DNA primers synthesized in Example 4 as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a synthetic

primer represented by the following sequence:
5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T) GA (C or T) (A or C) G (G or C) TAC-3'
(SEQ ID NO:22)

wherein I is inosine; and
a synthetic primer represented by the following sequence:
5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G) (C or T) GAA-3'
(SEQ ID NO:23)

wherein I is inosine, was carried out under the same conditions as in Example 1. The resulting PCR product was subcloned to the plasmid vector, pCR™II, in the same manner as in Example 2 to obtain a plasmid, p5S38. E. coli JM109 was transfected with the plasmid p5S38 to obtain transformant Escherichia coli JM109/p5S38.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), and the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and then the data of the nucleotide sequence obtained were read with DNASIS (Hitachi System Engineering Co., Japan).

Figure 12 shows a mouse pancreatic β-cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:19) and an amino acid sequence (SEQ ID NO:14) encoded by the isolated DNA based upon the nucleotide sequence of plasmid, p5S38. The underlined portions represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 12]. As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan), the

nucleotide sequence was converted into an amino acid sequence [Figure 12], and hydrophobicity plotting was carried out to confirm the presence of four hydrophobic regions [Figure 14]. Upon comparing the amino acid sequence with those encoded by p19P2 obtained in Example 2 and encoded by pG3-2 obtained in Example 4, furthermore, a high degree of homology was found as shown in Figure 13. As a result, it was strongly suggested that the mouse pancreatic β-cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 would recognize the same ligand as the human pituitary gland-derived G protein-coupled receptor protein encoded by p19P2 while the animal species from which the receptor protein encoded by p5S38 was derived was different from the species from which the receptor protein encoded by p19P2 was derived. It was also strongly suggested that the mouse pancreatic β-cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 would recognize the same ligand as the mouse pancreatic β-cell strain, MIN6-derived G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 and they would be analogous receptor proteins one another (so-called "subtype").

[Example 9]

Preparation of CHO cells which express phGR3

The plasmid phGR3 (Example 5) containing a cDNA encoding the full-length amino acid sequence of human pituitary receptor protein was digested with the restriction enzyme Nco I and electrophoresed on agarose gel and a fragment of about 1kb was recovered. Both ends of the recovered fragment were blunted with a DNA blunting kit (Takara Shuzo Co., Japan) and, after a SalI linker was added, the fragment was treated with SalI and inserted into the SalI site of pUC119 to provide plasmid S10. Then, S10 was treated with SalI and SacII to prepare a fragment of about 700 bp

(containing the N-terminal coding region). Then, a fragment of about 700 bp (containing the C-terminal coding region including initiation and termination codons) was cut out from phGR3 with Sac II and Nhe I. These two fragments were added to the animal cell expression vector plasmid pAKKO-111H (the vector plasmid identical to the pAKKO1.11 H described in Biochim. Biophys. Acta, Hinuma, S., et al., 1219 251-259, 1994) and a ligation reaction was carried out to construct a full-length receptor protein expression plasmid pAKKO-19P2.

E. coli transfected with pAKKO-19P2 was cultured and the pAKKO-19P2 plasmid DNA was mass-produced using QUIAGEN Maxi. A 20 μg portion of the plasmid DNA was dissolved in 1 ml of sterile PBS, and in a gene transfer vial (Wako Pure Chemical Ind.), the solution was vortexed well for liposome formation. This liposome, 125 μl, was added to CHOdhfr$^-$ cells previously subcultured at 1 x 10^6 cells per 10cm-dia. dish 24 hr and placed in fresh medium immediately before addition and overnight culture was carried out. After a further one-day culture in fresh medium, the medium was changed to a screening medium and the incubation was further carried out for a day. For efficient screening of transformants, subculture was carried out at a low cell density and only the cells growing in the screening medium were selected to establish a full-length receptor protein expression CHO cell line CHO-19P2.

[Example 10]

Confirmation of the amount of expression of the full-length receptor protein in the CHO-19P2 cell line at the transcription level

Using FastTrack Kit (Invitrogen), CHO cells transfected with pAKKO-19P2 according to the kit manual and mock CHO cells were used to prepare poly(A)$^+$RNA.

Using 0.02 μg of this poly(A)$^+$RNA, a cDNA was synthesized by means of RNA PCR Kit (Takara Shuzo, Co., Japan). The kind of primer used was a random 9mer and the total volume of the reaction mixture was 40 μl. As a negative control of cDNA synthesis, a reverse transcriptase-free reaction mixture was also provided. First, the reaction mixture was incubated at 30°C for 10 minutes to conduct an amplification reaction to some extent. Then, it was incubated at 42°C for 30 minutes to let the reverse transcription reaction proceed. The enzyme was inactivated by heating at 99°C for 5 minutes and the reaction system was cooled at 5°C for 5 minutes.

After completion of the reverse transcription reaction, a portion of the reaction mixture was recovered and after dilution with distilled water, extraction was carried out with phenol/chloroform and further with diethyl ether. The extract was subjected to precipitation from ethanol and the precipitate was dissolved in a predetermined amount of distilled water for use as a cDNA sample. This cDNA solution and the plasmid DNA (pAKKO-19P2) were serially diluted and using primers specific to full-length receptor protein, PCR was carried out. The sequences of the primers prepared according to the base sequence of the coding region of the full-length receptor protein were CTGACTTATTTTCTGGGCTGCCGC (SEQ ID NO:24) for 5' end and AACACCGACACATAGACGGTGACC (SEQ ID NO:25) for 3' end.

The PCR reaction was carried out in a total volume of 100 μl using 1 μM each of the primers, 0.5 μl of Taq DNA polymerase (Takara Shuzo Co., Japan), the reaction buffer and dNTPs accompanying the enzyme, and 10 μl of template DNA (cDNA or plasmid solution). First the reaction mixture was heat-treated at 94°C for 2 minutes for sufficient denaturation of the template DNA and subjected to 25 cycles of 95°C x 30 seconds, 65°C x 30

seconds, and 72°C x 60 seconds. After completion of the reaction, 10 µl of the reaction mixture was subjected to agarose gel electrophoresis and the detection and quantitative comparison of amplification products were carried out. As a result, a PCR product of the size (400 bp) predictable from the sequence of the cDNA coding for the full-length receptor protein was detected [Fig. 15]. In the lane of the PCR reaction mixture using the product of the reverse transcriptase-free transcription system as the template, no specific band was detected, thus extruding the possibility of its being a PCR product derived from the genomic DNA of CHO cells. Moreover, no specific band appeared in the lane of mock cells, either. Therefore, it was clear that the product was not derived from the mRNA initially expressed in CHO cells [Fig. 15].

[Example 11]

Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a rat whole brain extract

A crude peptide fraction was prepared from rat whole brain by the following procedure. The rat whole brain enucleated immediately after sacrifice was frozen in liquefied nitrogen and stored at -80°C. The frozen rat whole brain, 20 g (the equivalent of 10 rats) was finely divided and boiled in 80 ml of distilled water for 10 minutes. After the boiled tissue was quenched on ice, 4.7 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (20,000 rpm, 6 min.). The homogenate was stirred overnight and then centrifuged (10,000 rpm, 20 min.) to separate the supernatant. The sediment was homogenized in 40 ml of 1.0 M acetic acid and centrifuged again to recover the supernatant. The supernatants were pooled, diluted in 3 volumes of acetone, allowed to stand on ice for 30 minutes, and

centrifuged (10,000 rpm, 20 min.) to recover the supernatant. The recovered supernatant was evaporated to remove acetone. To the resulting acetone-free concentrate was added 2 volumes of 0.05% trifluoroacetic acid(TFA)/H_2O and the mixture was applied to a reversed-phase C18 column (Prep C18 125Å, Millipore). After application of the supernatant, the column was washed with 0.05% TFA/H_2O, and gradient elution was carried out with 10%, 20%, 30%, 40%, 50%, and 60% CH_3CN/0.05%TFA/H_2O. The fractions were respectively divided into 10 equal parts and lyophilized. The dried sample derived from one animal equivalent of rat whole brain was dissolved in 20 µl of dimethyl sulfoxide (DMSO) and suspended in 1 ml of Hank's balanced saline solution (HBSS) supplemented with 0.05% bovine serum albumin (BSA) to provide a crude peptide fraction.

The full-length receptor protein-expressed CHO cells and mock CHO cells were seeded in a 24-well plate, 0.5 x 10^5 cells/well, and cultured for 24 hours. Then, [^{3}H] arachidonic acid was added at a final concentration of 0.25µCi/well. Sixteen (16) hours after addition of [^{3}H] arachidonic acid, the cells were rinsed with 0.05% BSA-HBSS and the above-mentioned crude peptide fraction was added, 400 µl/well. The mixture was incubated at 37°C for 30 minutes and a 300 µl portion of the reaction mixture (400 µl) was added to a scintillator (4 ml) and the amount of [^{3}H] arachidonic acid metabolite released into the reaction mixture was determined with a scintillation counter. As a result, an arachidonic acid metabolite-releasing activity specific to the full-length receptor protein expressed CHO cells (CHO-19P2) was detected in the 30% CH_3CN fraction of the eluate [Fig. 16].

[Example 12]

Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a bovine hypothalamus extract

A crude peptide fraction was prepared from 360 g (the equivalent of 1 animals) of bovine brain tissue including hypothalamus in the same manner as in Example 11. A dried peptide sample per 0.05 animal was dissolved in 40 µl of DMSO and suspended in 2 ml of 0.05% BSA-HBSS and the detection of arachidonic acid metabolite-releasing activity was attempted in the same manner as in Example 11. As a result, the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the fraction eluted with 30% CH_3CN from a C18 column to which the crude bovine hypothalamus peptide fraction had been applied [Fig. 17].

[Example 13]

Preparation of the activity (peptide) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells by purification from bovine hypothalamus

A typical process for harvesting the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line by purification from bovine hypothalamus is now described. A frozen bovine brain tissue specimen including hypothalamus, 4.0 kg (the equivalent of 80 animals) was ground and boiled in 8.0 L of distilled water for 20 minutes. After quenching on ice, 540 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (10,000 rpm, 12 min.). The homogenate was stirred overnight and then centrifuged (9,500 rpm, 20 min) to recover a supernatant. The sediment was suspended in 4.0 L of 1.0 M acetic acid and homogenized with the Polytron and centrifuged again to recover a further supernatant.

The supernatants were pooled and TFA was added at a final concentration of 0.05%. The mixture was applied to reversed-phase C18 (Prep C18 125Å, 160 ml; Millipore) packed in a glass column. After addition, the column was washed with 320 ml of 0.05% TFA/H_2O and 3-gradient elution was carried out with 10%, 30%, and 50% CH_3CN/0.05% TFA/H_2O. To the 30% CH_3CN/0.05% TFA/H_2O fraction was added 2 volumes of 20 mM CH_3COONH_4/H_2O and the mixture was applied to the cation exchange column HiPrep CM-Sepharose FF (Pharmacia). After the column was washed with 20 mM CH_3COONH_4/10% CH_3CN/H_2O, 4-gradient elution was carried out with 100 mM, 200 mM, 500 mM, and 1000 mM CH_3COONH_4/10% CH_3CN/H_2O. In the 200 mM CH_3COONH_4 fraction, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 was detected. Therefore, this fraction was diluted with 3 volumes of acetone, centrifuged for deproteination, and concentrated in an evaporator. To the concentrated fraction was added TFA (final concentration 0.1%) and the mixture was adjusted to pH 4 with acetic acid and applied to 3 ml of the reversed-phase column RESOURCE RPC (Pharmacia). Elution was carried out on a concentration gradient of 15%-30% CH_3CN. As a result, activity to specifically promote the release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the 19%-21% CH_3CN fraction. The active fraction eluted from RESOURCE RPC was lyophilized, dissolved with DMSO, suspended in 50 mM MES pH 5.0/10% CH_3CN, and added to 1 ml of the cation exchange column RESOURCE S. Elution was carried out on a concentration gradient of 0 M-0.7 M NaCl. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the 0.32 M-0.46 M NaCl fraction. The active eluate from RESOURCE S was lyophilized,

dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to reversed-phase column C18 218TP5415 (Vydac), and elution was carried out on a concentration gradient of 20%-30% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the three fractions 22.5%, 23%, and 23.5% CH_3CN (these active fractions are designated as P-1, P-2, and P-3) [Fig. 18]. Of the three active fractions, the 23.5% CH_3CN fraction (P-3) was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to the reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 22%-25% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was converged by recovery in one elution peak obtained with 23% CH_3CN [Fig. 19]. The active peak fraction from the reverse-phased column diphenyl 219TP5415 was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to the reversed-phase column μRPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a gradient of 22%-23.5% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the two peaks eluted with 23.0% and 23.2% CH_3CN [Fig. 20].

[Example 14]

Determination of the amino acid sequence of the peptide having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

The amino acid sequence of the peptide (P-3) having activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as

purified in Example 13 was determined. The fraction showing peak activity from the reversed-phase μRPC C2/C18 SC 2.1/10 was lyophilized and dissolved in 20 μl of 70% CH_3CN and analyzed for amino acid sequence with the peptide sequencer (ABI.491). As a result, the sequence defined by SEQ ID NO:26 was obtained. However, the 7th and 19th amino acids were not determined by only the analysis of amino acid sequence.

[Example 15]

Preparation of the active substance (peptide) which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

Of the three active fractions obtained with Vydac C18 218TP5415 in Example 13, the active fraction (P-2) eluted with 23.0% CH_3CN was further purified. This active fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/dH_2O, and added to reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 21.0%-24.0% CH_3CN. As a result, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in a peak eluted with 21.9% CH_3CN. This fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/dH_2O, and added to reversed-phase μRPC C2/C18 SC 2.1/10 (Pharmacia), and then elution was carried out on a CH_3CN gradient of 21.5%-23.0%. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells converged in one peak eluted with 22.0% CH_3CN [Fig. 21].

[Example 16]

Determination of the amino acid sequence of the peptide (P-2) purified from bovine hypothalamus which specifically promotes release of arachidonic

acid metabolites from CHO-19P2 cells

The amino acid sequence of the peptide (P-2) having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 15 was determined. The active peak fraction from the reversed-phase column μRPC C2/C18 SC 2.1/10 was lyophilized, dissolved in 20 μl of 70% CH_3CN, and analyzed for amino acid sequence with the peptide sequencer (ABI, 492) (SEQ ID NO:27).

[Example 17]

Preparation of a poly(A)$^+$RNA fraction from bovine hypothalamus and synthesis of a cDNA

Using Isogen (Nippon Gene), total RNA was prepared from one animal equivalent of bovine hypothalamus. Then, using Fast Track (Invitrogen), a poly(A)$^+$RNA fraction was prepared. From 1 μg of this poly(A)$^+$RNA fraction, cDNA was synthesized using 3' RACE system (GIBCO BRL) and Marathon cDNA amplification kit (Clontech) according to the manuals and dissolved in 20 and 10 μl, respectively.

[Example 18]

Acquisition of cDNA coding for the amino acid sequence established in Example 14

To obtain a cDNA coding for a polypeptide comprising the amino acid sequence established in Example 14, the acquisition of a base sequence coding for SEQ ID NO:28 was attempted in the first place. Thus, primers P5-1 (SEQ ID NO:29), P3-1 (SEQ ID NO:30), and P3-2 (SEQ ID NO:31) were synthesized. (In the Sequence Table, I represents inosine). Using 0.5 μl of the cDNA prepared by 3' RACE in Example 17 as a template and EXTaq (Takara Shuzo Co., Japan) as DNA polymerase, 2.5 μl of accompanying buffer, 200 μM of accompanying dNTP, and primers P5-1 and P3-1 were added each at a final concentration of 200 nM, with water added to make 25 μl, and after treatment for one minute

at 94°C, the cycle of 98°C x 10 seconds, 50°C x 30 seconds, 68°C x 10 seconds was repeated 30 times. This reaction mixture was diluted 50-fold with tricin-EDTA buffer and using 2.5 µl of the dilution as a template and the primer combination of P5-1 and P3-2, the reaction was carried out in otherwise the same manner as described above. As the thermal cycler, Gene Amp 9600 (Perkin Elmer) was used. The amplification product was subjected to 4% agarose gel electrophoresis and ethidium bromide staining and a band of about 70 bp was cut out and subjected to thermal fusion, phenol extraction, and ethanol precipitation. The recovered DNA was subcloned into plasmid vector PCR™II according to the manual of TA Cloning kit (Invitrogen). The vector was then introduced into E. coli JM109 and the resultant transformant was cultured in ampicillin-containing LB medium. The plasmid obtained with an automatic plasmid extractor (Kurabo) was reacted according to the manual of Dye Terminator Cycle Sequencing Kit (ABI) and decoded with a fluorescent automatic DNA sequencer (ABI). As a result, the sequence shown in Fig. 22 was obtained and confirmed to be part of the base sequence coding for SEQ ID NO:28.

[Example 19]

Acquisition of a bioactive polypeptide cDNA by RACE using the sequence established in Example 18

First, for amplification (5' RACE) of the sequence at 5' end, the two primers PE (SEQ ID NO:32) and PDN (SEQ ID NO:33) were synthesized by utilizing the sequence shown in Fig. 22. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold with tricin-EDTA buffer. Then, in the same manner as Example 2, a reaction mixture was prepared using 2.5 µl of the dilution and a combination of the adapter primer AP1 accompanying the kit and the primer PE and after treatmenet for one minute at 94°C,

the cycle of 98°C x 10 seconds and 68°C x 5 minutes was repeated 30 times. This reaction system was further diluted 50-fold with tricin-EDTA buffer and using 2.5 µl of the dilution as a template and the changed primer combination of AP1 and PDN, the reaction was conducted at 94°C for one minute, followed by 4 cycles of 94°C x 1 minute, 98°C x 10 seconds and 72°C x 5 minutes, 4 cycles of 98°C x 10 seconds and 70°C x 5 minutes, and 26 cycles of 98°C x 10 seconds and 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 150 bp was cut out and centrifugally filtered through a centrifugal filter tube (Millipore), extracted with phenol, and precipitated from ethanol. The recovered DNA was subcloned into plasmid vector PCR™II according to the manual of TA Cloning Kit (Invitrogen). The vector was then introduced into E. coli JM109 and the resulting transformant was cultured and the sequence of the inserted cDNA fragment was analyzed as in Example 18. As a result, the sequence shown in Fig. 23 was obtained. Based on this sequence, primers FB (SEQ ID NO:34) and FG (SEQ ID NO:35) were synthesized and the 3' sequence was cloned (3' RACE). Using the same template as that for 5' RACE in the same quantity and the combination of the accompanying adapter primer AP1 with the primer FC, PCR was carried out at 94°C for 1 minute, followed by 5 cycles of 98°C x 10 seconds and 72°C x 5 minutes, 5 cycles of 98°C x 10 seconds and 70°C x 5 minutes, and 25 cycles of 98°C x 10 seconds and 68°C x 5 minutes. Then, using 2.5 µl of a 50-fold dilution of this reaction mixture in tricin-EDTA buffer as the template and the combination of the accompanying primer AP2 with the primer FB, the reaction was further conducted at 94°C for one minute, followed by 4 cycles of 98°C x 10 seconds and 72°C x 5 minutes, 4 cycles of 98°C x 10 seconds and 70°C x 5

minutes, and 27 cycles of 98°C x 10 seconds and 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 400 bp was cut out and the DNA was recovered as in 5'-RACE. This DNA fragment was subcloned into plasmid vector pCR™II and introduced into E. coli JM109 and the sequence of the inserted cDNA fragment in the resulting transformant was analyzed. From the results of 5' RACE and 3' RACE, the DNA sequence [Fig. 24] coding for the complete coding region of the bioactive polypeptide defined by SEQ ID NO:1 was established. Thus, in Fig. 24 (a) and (b), the base^{134} is G, the base^{184} is T or C, and the base^{245} was T or C.

The cDNA shown in Fig. 24 was the cDNA encoding a polypeptide consisting of 98 amino acids. The fact that the amino acids in 1 - 22-positions comprise a cluster of hydrophobic amino acids taken together with the fact that the N-terminal region of the active peptide begins with Ser in 23-position as shown in Example 14 suggested that the amino acids 1-22 represent a secretion signal sequence. On the other hand, the Gly Arg Arg Arg sequence in 54-57 positions of the polypeptide was found to be a typical amino acid sequence motif which exists in the event of cleavage of a bioactive peptide. As it is the case with this cleavage motif, it is known that because of the presence of Gly, the C-terminus of the product peptide is frequently amidated.

The P-3 N-terminal sequence data of Example 14 and P-2 N-terminal sequence data in Example 16 coupled with this GlyArgArgArg sequence suggest that at least the same of the bioactive peptides cut out from the polypeptide encoded by this cDNA are defined by SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43 or SEQ ID NO:44.

[Example 20]

Acquisition of a DNA fragment comprising the full coding region of bovine-derived bioactive polypeptide cDNA by PCR

Using the cDNA prepared with Marathon cDNA amplification kit in Example 17 as a template, a DNA fragment including the entire coding region of bioactive polypeptide cDNA was constructed. First, based on the sequence of cDNA elucidated in Example 19, two primers having base sequences defined by SEQ ID NO:42 and SEQ ID NO:43, respectively, were synthesized.

BOVF

5'-GTGTCGACGAATGAAGGCGGTGGGGGCCTGGC-3' (SEQ ID NO:36)

BOVR (24 mer)

5'-AGGCTCCCGCTGTTATTCCTGGAC-3' (SEQ ID NO:37)

BOVF contains the initiation codon of bioactive polypeptide cDNA and is a sense sequence corresponding to -2 - +22 (A of the initiation codon ATG being reckoned as +1) with restriction enzyme SalI site added. On the other hand, BOVR is an antisense sequence corresponding to +285 - +309 which includes the termination codon of bioactive polypeptide cDNA.

The PCR was conduced as follows. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold in tricin-EDTA buffer and using 2.5 μl of the dilution, a reaction mixture was prepared as in Example 2 and subjected to 94°C x 1 minute, 3 cycles of 98°C x 10 seconds and 72°C x 5 minutes, 3 cycles of 98°C x 10 seconds and 70°C x 5 minutes, and 27 cycles of 98°C x 10 seconds and 68°C x 5 minutes. The amplification product was subjected to 2% agarose electrophoresis and ethidium bromide staining and a band of about 320 bp was cut out. The DNA was recovered and subcloned in plasmid vector pCR™II as in Example 3. The vector was introduced into Escherichia coli JM109 to provide the transformant

E. coli JM109/pBOV3. The sequence of the cDNA fragment inserted in the transformant was then analyzed. As a result, this DNA fragment was confirmed to be a fragment covering the entire coding region of the bioactive polypeptide cDNA.

[Example 21]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH2 (19P2-L31)

1) Synthesis of Ser(Bzl)-Arg(Tos)-Ala-His(Bom)-Gln-His(Bom)-Ser(Bzl)-Met-Glu(OcHex)-Ile-Arg(Tos)-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin

The reactor of a peptide synthesizer (Applied Biosystems 430A) was charged with 0.71 g (0.5 mmole) of commercial p-methyl-BHA resin (Applied Biosystems, currently Perkin Elmer). After wetting with DCM, the initial amino acid Boc-Phe was activated by the HOBt/DCC method and introduced into the p-methyl-BHA resin. The resin was treated with 50% TFA/DCM to remove Boc and make the amino group free and neutralized with DIEA. To this amino group was condensed the next amino acid Boc-Arg (Tos) by the HOBt/DCC method. After the absence of unreacted amino function was verified by ninhydrin test, a sequential condensation of Boc-Gly, Boc-Val, Boc-Pro, Boc-Arg(Tos), Boc-Ile, Boc-Gly, Boc-Arg(Tos), Boc-Gly, Boc-Ala, Boc-Tyr(Br-Z) was carried out. The Boc-Ala and Boc-Tyr (Br-Z), the condensation of which was found insufficient by ninhydrin test, was recondensed to complete the reaction. The resin was dried and a half of the resin was withdrawn. To the remainder, Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro, Boc-Thr(Bzl), Boc-Arg(Tos), Boc-

Ile, Boc-Glu(OcHex), Boc-Met, Boc-Ser(Bzl), Boc-His(Bom), Boc-Gln, Boc-His(Bom), Boc-Ala, Boc-Arg(Tos), Boc-Ser(Bzl) were serially condensed and recondensed until sufficient condensation was confirmed by ninhydrin test. After introduction of the full sequence of amino acids of 19P2-L31, the resin was treated with 50% TFA/DCM to remove Boc groups on the resin and, then, dried to provide 1.28 g of the peptide resin.

2) Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH2(19P2-L31)

In a Teflon hydrogen fluoride reactor, the resin obtained in 1) was reacted with 3.8 g of p-cresol, 1 ml of 1,4-butanedithiol, and 10 ml of hydrogen fluoride at 0°C for 60 minutes. The hydrogen fluoride and 1,4-butanedithiol (1 ml) were distilled off under reduced pressure and the residue was diluted with 100 ml of diethyl ether, stirred, filtered through a glass filter, and the fraction on the filter was dried. This fraction was suspended in 50 ml of 50% acetic acid/H_2O and stirred to extract the peptide. After separation of the resin, the extract was concentrated under reduced pressure to about 5 ml and chromatographed on Sephadex G-25 (2 x 90 cm). Development was carried out with 50% acetic acid/H_2O and the 114 ml - 181 ml fraction was pooled and lyophilized to recover 290 mg of white powders containing 19P2-L31. The powders were applied to a reversed-phase column of LiChroprep RP-18 (Merck) and repeatedly purified by gradient elution using 0.1% TFA/H_2O and 0.1% TFA-containing 30% acetonitrile/H_2O. The fraction eluted at about 25% acetonitrile was pooled and lyophilized to provide 71 mg of white powders.

Mass spectrum $(M+H)^+$ 3574.645

HPLC elution time 18.2 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/H_2O)

B (0.1% TFA-containing 50 (% acetonitrile/H_2O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 22]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met(O)-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH2(19P2-L31(O))

In 20 ml of 5% acetic acid/H_2O was dissolved 6 mg of synthetic 19P2-L31 and the Met only was selectively oxidized with 40 µl of 30% H_2O_2. After completion of the reaction, the reaction mixture was immediately applied to a reversed-phase column of LiChroprep RP-18 (Merck) for purification to provide 5.8 mg of the objective peptide.

Mass spectrum $(M+H)^+$ 3590.531

HPLC elution time 17.9 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/H_2O)

B (0.1% TFA-containing 50% aceto nitrile/H_2O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 23]

Synthesis of Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH2(19P2-L20)

To the resin subjected to condensations up to Boc-Tyr(Br-Z) in Example 21-1) was further condensed with Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro and Boc-Thr(Bzl) serially in the same manner to provide 1.14 g of Boc-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-

Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin. This resin was treated with hydrogen fluoride and purified column chromatography in the same manner as Example 21-2) to provide 60 mg of white powders.

Mass spectrum $(M+H)^+$ 2242.149

HPLC elution time 10.4 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA-containing 15% aceto nitrile/H_2O)

B (0.1% TFA-containing 45% aceto nitrile/H_2O)

Linear gradient elution from A to B (15 min.)

Flow rate: 1.0 ml/min.

[Example 24]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L31)

The activity of the peptide (19P2-L31) synthesized in Example 21 to specifically release arachidonic acid metabolites from CHO-19P2 cells was assayed in the same manner as Example 11. The synthetic peptide was dissolved in degassed distilled water at a concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS and the activity to promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [^{3}H]arachidonic acid metabolites as the indicator. As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10^{-12}M - 10^{-6}M [Fig. 25]. When the arachidonic acid metabolite-releasing activity of peptide 19P2-L31(O), i.e. the methionine-oxidation product of 19P2-L31 synthesized in Example 22, was compared with that of 19P2-L31, it was found that the activity of 19P2-L31(O) was equivalent to the activity of 19P2-L31 as can be

seen from Fig. 26.

[Example 25]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L20)

The activity of the synthetic equivalent (19P2-L20) of natural peptide P-2 as synthesized in Example 23 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was determined as in Example 11. Thus, the synthetic peptide was dissolved in degassed distilled water at a final concentration of 10^{-3}M and this solution was serially diluted with 0.05% BAS-HBSS. The activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [^{3}H]arachidonic acid metabolites as the indicator.

As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10^{-12} - 10^{-6}M in nearly the same degree as 19P2-L31 [Fig. 27].

[Example 26]

Analysis of the coding region base sequence of bovine genomic DNA

pBOV3 was digested with restirction enzyme EcoRI and after fractionation by agarose gel electrophoresis, the DNA corresponding to the cDNA fragment was recovered to prepare a probe. This DNA was labeled with ^{32}P using a multiprime DNA labeling kit (Amersham). About 2.0×10^{6} phages of Bovine Genomic Library (Clontech BL1015j) constructed using cloning vector EMBL3 SP6/T7 and Escherichia coli K802 as the host were seeded in an LB agar plate and cultured overnight for plaque formation. The plaques were transferred to a nitrocellulose filter and after alkaline modification and neutralization, heat-treated (80°C, 2 hours) to inactivate the DNA. This filter was incubated with the labeled probe in 50% formamide-Hybri

buffer (50% formamide, 5 x Denhardt solution, 4 x SSPE, 0.1 mg/ml heat-denatured salmon sperm DNA, 0.1% SDS) at 42°C overnight for hybridization. After this hybridization, the filter was washed with 2 x SSC, 0.1% SDS at room temperature for 1.5 hours, and further washed in the same buffer at 55°C for 30 minutes. Detection of the clone hybridizing with the probe was carried out on Kodak X-ray film (X-OMAT™AR) after 4 days of exposure using a sensitization screen at -80°C. After development of the film, the film was collated with plate positions and the phages which had hybridized were recovered. Then, plating and hybridization were repeated in the same manner for cloning of the pharges.

The cloned phages were prepared on a large scale by the plate lysate method and the phage DNA was extracted. Then, cleavage at the restriction enzyme SalI and BamHI cleavage sites at both ends of the cloning site of the vector and detection of the inserted fragment derived from bovine genomic DNA was carried out by 1.2% agarose gel electrophoresis [Fig. 28]. As a result, in the case of BamHI digestion, 3 fragments were detected in addition to the bands derived from the phages. In the case of SalI digestion, one band overlapping the phage band was detected. The SalI-digested fragment being considered to harbor the full length and in order to subclone this fragment into a plasmid vector, it was ligated to BAP (E. coli-derived alkaline phosphatase)-treated plasmid vector pUC18 (Pharmacia) and introduced into E. coli JM109. From this microorganism, a genome-derived SalI fragment-inserted plasmid DNA was prepared on a production scale and the base sequence in the neighborhood of its coding region was analyzed using Perkin Elmer Applied Biosystems 370A fluorecent sequencer and the same manufacturer's kit. As a

result, the sequence shown in Fig. 29 was obtained. Comparison with the coding region of cDNA reveals that because of its being derived from genomic DNA, the coding region is divided in two by a 472 bp intron [Fig. 30]. Fig. 31 and SEQ ID NO:44 present the amino acid sequence predicted from this bovine genome coding region (excluding the intron region).

[Example 27]

Preparation of rat medulla oblongata poly(A^+)RNA fraction and synthesis of cDNA

Using Isogen (Nippon Gene), total RNA was prepared from the dorsal region of rat medulla oblongata and using FastTrack (Invitrogen), poly$(A)^+$RNA fraction was prepared. To 5 µg of this poly$(A)^+$RNA was added the primer random DNA hexamer (BRL) and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 12 µl of distilled water. In addition, from 1 µg of this poly$(A)^+$RNA, a cDNA was synthesized using Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10 µl of DW.

[Example 28]

Acquisition of rat bioactive polypeptide cDNA by RACE

To obtain the full coding region of rat bioactive polypeptide cDNA, an experiment was performed in the same manner as the acquisition of bovine cDNA. First, PCR was carried out using the same primers P5-1 (SEQ ID NO:29) and P3-1 (SEQ ID NO:30) as used in Example 18 as primers and the complementary DNA synthesized in Example 27 using the primer random DNA hexamer (BRL) and Moloney mouse leukemia reverse transcriptase (BRL) as a template. The reaction system was composed of 1.25 µl of the template cDNA, 200 µM of dNTP, 1 µM each of the primers, ExTaq (Takara Shuzo Co., Japan) as DNA

polymerase, and 2.5 µl of the accompanying buffer, with a sufficient amount of water to make a total of 25 µl. The reaction was carried out at 94°C for 1 minute, followed by 40 cycles of 98°C x 10 seconds, 50°C x 30 seconds, and 72°C x 5 seconds, and the reaction mixture was then allowed to stand at 72°C for 20 seconds. The thermal cycler used was GeneAmp2400 (Perkin Elmer). The amplification product was subjected to 4% agarose gel electrophoresis and ethidium bromide staining and the band of about 80 bp was cut out. Then, in the manner described in Example 19, the DNA was recovered, subcloned into plasmid vector pCR™II, and introduced into E. coli JM109, and the inserted cDNA fragment was sequenced. As a result, a partial sequence of rat bioactive polypeptide could be obtained. Based on this sequence, two primers, namely RA (SEQ ID NO:45) for 3' RACE and RC (SEQ ID NO:46) for 5' RACE were synthesized and 5' and 3' RACEs were carried out.

RA:5'-CARCAYTCCATGGAGACAAGAACCCC-3'

(where R means A or G; Y means T or G) (SEQ ID NO:45)

RC:5'-TACCAGGCAGGATTGATACAGGGG-3'

(SEQ ID NO:46)

As a template, the template synthesized using Marathon cDNA amplification kit (Clontech) in Example 27 was diluted 40-fold with the accompanying tricin-EDTA buffer and 2.5 µl of the dilution was used. As primers, RA and the adapter primer AP1 accompanying the kit were used for 3' RACE, and RC and AP1 for 5' RACE. The reaction mixture was prepared in otherwise the same manner as above. The reaction conditions were 94°C x 1 minute, 5 cycles of 98°C x 10 seconds and 72°C x 45 seconds, 3 cycles of 98°C x 10 seconds and 70°C x 45 seconds, and 40 cycles of 98°C x 10 seconds and 68°C x 45 seconds. As a result, a band of about 400 bp was obtained from 3' RACE and bands of about 400 bp and 250 bp from 5' RACE. These bands were recovered in the

same manner as above and using them as templates and the primers used in the reaction, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, the sequence up to poly A could be obtained from the region considered to be the 5' noncoding region.

[Example 29]

Acquisition of the full-length cDNA of rat bioactive polypeptide by PCR

Based on the sequence obtained in Example 28, two primers, viz. rF for the region including the initiation codon (SEQ ID NO:47) and rR for the 3' side from the termination codon (SEQ ID NO:48), were synthesized to amplify the fragment including the full-length cDNA.

rF:5'-GGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:47)

rR:5'-AGCAGAGGAGAGGGAGGGTAGAGGA-3' (SEQ ID NO:48)

Using the cDNA prepared using Moloney mouse leukemia reverse transcriptase in Example 27 as a template and ExTaq (Takara Shuzo Co., Japan), PCR was carried out by repeating 40 cycles of 95°C x 30 seconds and 68°C x 60 seconds. The amplification product was subjected to agarose gel electrophoresis and ethidium bromide staining and a band of about 350 bp was cut out. The DNA was recovered, subcloned into plasmid vector pCR™II, and introduced into E. coli JM109 as in Example 19. The plasmid was extracted from the transformant and the base sequence was determined. As a result, E. coli JM 109/pRAV3 having the full-length cDNA of rat bioactive polypeptide was obtained [Fig. 32].

[Example 30]

Synthesis of cDNA from the human total brain poly(A)$^+$RNA fraction

From 1 μg of human total brain poly(A)$^+$RNA fraction (Clontech), cDNA was synthesized with Marathon

cDNA amplification kit (Clontech) according to the manual and dissolved in 10 µl. In addition, the random DNA hexamer (BRL) was added as primer to 5 µg of the same poly(A)$^+$RNA fraction and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 30 µl of Tris EDTA.

[Example 31]

Acquisition of human bioactive polypeptide cDNA by RACE

From the amino acid sequence of rat bioactive polypeptide established in Example 28 [Fig. 33], the well-preserved regions of rat and bovine polypeptides were selected and the following 3 primers R1 (SEQ ID NO:49), R3 (SEQ ID NO:50), and R4 (SEQ ID NO:51) were synthesized. Then, amplification of the region flanked by them was attempted by PCR using human cDNA as a template. Referring to Fig. 33, "bovine. aa" represents the amino acid sequence of bovine polypeptide, "bovine. seq" represents the base sequence of the DNA coding for bovine polypeptide, and "rat. seq" represents the base sequence of the DNA coding for rat polypeptide.

R1:5'-ACGTGGCTTCTGTGCTTGCTGC-3' (SEQ ID NO:49)
R3:5'-GCCTGATCCCGCGGCCCGTGTACCA-3' (SEQ ID NO:50)
R4:5'-TTGCCCTTCTCCTGCCGAAGCGGCCC-3' (SEQ ID NO:51)

The cDNA prepared using Marathon cDNA amplification kit (Clontech) in Example 30 was diluted 30-fold with tricin-EDTA buffer and 0.25 µl of the dilution was used as a template. The reaction mixture was composed of 200 µM of dNTP, 0.2 µM each of the primers R1 and R4, a 50:50 mixture of Taq Start Antibody (Clontech) and DNA polymerase ExTaq (Takara Shuzo Co., Japan), 2.5 µl of the accompanying buffer, and a sufficient amount of water to make a total volume

of 25 µl. The reaction conditions were 94°C x 1 minute, followed by 42 cycles of 98°C x 10 seconds and 68°C x 40 seconds, and 1 minute of standing at 72°C. Then, using 1 µl of a 100-fold dilution of the above reaction mixture in tricine-EDTA buffer as a template, the same reaction mixture as above except that the primer combination was changed to R1 and R3 was prepared and PCR was carried out in the sequence of 94°C x 1 minute and 25 cycles of 98°C x 10 seconds and 68°C x 40 seconds. The amplification product was subjected to 4% agarose gel electrophoresis and ethidium bromide staining. As a result, a band of about 130 bp was obtained as expected. This band was recovered in the same manner as in Example 28 and using the recovered fragment as a template, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, a partial sequence of human bioactive polypeptide could be obtained. Therefore, based on this sequence, primers HA (SEQ ID NO:52) and HB (SEQ ID NO:53) were synthesized for 3' RACE and primers HE (SEQ ID NO:54) and HF (SEQ ID NO:55) for 5' RACE and 5' and 3' RACEs were carried out.

HA:5'-GGCGGGGGCTGCAAGTCGTACCCATCG-3' (SEQ ID NO:52)
HB:5'-CGGCACTCCATGGAGATCCGCACCCCT-3' (SEQ ID NO:53)
HE:5'-CAGGCAGGATTGATGTCAGGGGTGCGG-3' (SEQ ID NO:54)
HF:5'-CATGGAGTGCCGATGGGTACGACTTGC-3' (SEQ ID NO:55)

As the template, 2.5 µl of a 20-fold dilution of the cDNA prepared in Example 30 in tricin-EDTA buffer was used. For the initial PCR, reaction mixtures were prepared in the same manner as above except that HA and adapter primer AP1 were used for 3' RACE and HE and AP1 for 5' RACE. The reaction sequence was 94°C x 1 minute, 5 cycles of 98°C x 10 seconds and 72°C for 35 seconds, 5 cycles of 98°C x 10 seconds and 70°C x 35 seconds, and 40 cycles of 98°C x 10 seconds and 68°C x 35 seconds. Then, using 1 µl of a 100-fold dilution of

this reaction mixture in tricin-EDTA buffer as a template, a second PCR was carried out in the same cycles as the first PCR. However, the reaction mixture was prepared using primers HB and AP1 for 3' RACE or HF and AP2 for 5' RACE and Klen Taq (Clontech) as DNA polymerase and the accompanying buffer. As a result, a band of about 250 bp was obtained from 3' RACE and a band of about 150 bp from 5'-RACE. These bands were sequenced by the same procedure as above and using them in combination with the partial sequence obtained previously, the sequence from the region presumed to be 5'-noncoding region to poly(A) of human bioactive polypeptide was obtained.

[Example 32]

Acquisition of human bioactive polypeptide full-length cDNA by PCR

Based on the sequence obtained in Example 31, two primers 5H (SEQ ID NO:56) and 3HN (SEQ ID NO:57) were synthesized for amplification of a fragment including full-length cDNA.

5H:5'-GGCCTCCTCGGAGGAGCCAAGGGATGA-3' (SEQ ID NO:56)

3HN:5'-GGGAAAGGAGCCCGAAGGAGAGGAGAG-3' (SEQ ID NO:57)

Using 2.5 μl of the cDNA prepared using Moloney mouse leukemia reverse transcriptase (BRL) in Example 30 as a template and the reaction mixture prepared using Klen Taq DNA polymerase (Clontech), the PCR reaction was conducted in the sequence of 94°C x 1 minute and 40 cycles of 98°C x 10 seconds and 68°C x 30 seconds. The fragment of about 360 bp obtained was recovered and subcloned (pCR™ 2.1 was used as the vector) in the same manner as Example 29. The plasmid was recovered and its base sequence was determined. As a result, E. coli JM109/pHOV7 harboring the human bioactive polypeptide full-length cDNA was obtained [Fig. 34]. In regard to the amino acid sequence of the translation region, a comparison was made between this

human bioactive polypeptide and the bovine polypeptide shown in Example 20 or the rat polypeptide in Example 29 [Fig. 35].

[Example 33]

Acquisition and sequencing of a DNA including the ligand polypeptide coding region from the murine genomic DNA

Based on the sequence (Fig. 32) of the cDNA coding for the rat ligand polypeptide obtained in Example 29, the following two primers were synthesized.

rFBG: 5'-AGATTGGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:61)

rRSA: 5'-GTCGACTCAGCAGCACTGTCTTCTCGAGCTG-3' (SEQ ID NO:62)

Using the above two primers and, as a template, 0.5 ng of murine genomic DNA (Mouse BALB/c genomic DNA), a PCR amplification was carried out.

The reaction components used were: 200 nM each synthetic DNA primers, 0.5 ng template DNA, 0.5 µl of 0.25 mM dNTPs ExTaq polymerase, and enzyme-attached buffer, total volume 50 µl. Using a thermal cycler (Perkin-Elmer), an amplification reaction was carried out in 30 cycles of 30 seconds at 95°C and 60 seconds at 67°C. Identification of the amplification product was made by 1.2% agarose gel electrophoresis and ethidium bromide staining, and a band of about 1 kb was recovered and subcloned using TA Cloning Kit (Invitrogen). This ligation mixture was used to transform Escherichia coli JM109 and clones harboring the inserted fragment were selected in LB agar containing ampicillin and X-gal. A white-colored clone was picked out to obtain a transformant Escherichia coli JM109/pmGB3. This clone was cultured overnight in ampicillin-containing LB medium and the plasmid DNA was prepared using an automatic plasmid extraction apparatus. Using a portion of the DNA thus prepared, a

sequencing reaction was carried out using ABI Dye Terminator Cycle Sequencing Kit (ABI), and after decoding with a fluorescent automated sequencer, the nucleotide sequence data was analyzed with DNASIS (Hitachi System Engineering) [Fig. 36]. The underlined sequences correspond to the primers.

The nucleotide sequence thus determined was compared with the sequence of SEQ ID NO:58, 59, or 60. As a result, it was found that the DNA fragment in the plasmid pmGB3 harbored by Escherichia coli JM109/pmGB3 codes for a novel murine ligand polypeptide [Fig. 37].

[Example 34]

Acquisition and Sequencing of the full-length translated region DNA of the ligand polypeptide coding region of mouse genomic DNA

The DNA fragment contained in the plasmid pmGB3 as obtained in Example 33 was prepared and sent to Genome Systems with a request for hybridization screening from Mouse ES129/SuJ BAC library using the fragment as the probe (Catalogue BAC, 4921). Fromt the BAC clone received from Genome Systems, the DNA fragment containing the objective ligand peptide coding region was subcloned in the EcoRI site of pUC18 Vector and JM109/pmGFEI was obtained. Then, the sequence of the region of interest was similarly determined. The determined sequence (Fig. 38) coded for the indicated amino sequence. The sequence in parentheses differs from that obtained in Example 33. While the latter was the PCR primer used, the sequence obtained this time was shorter by 5 residues.

[Example 35]

Construction of a targeting vector for production of knockout mice

The targeting vector was constructed using pmGFE1 obtained in Example 34 and pGT-N28 (NEB). The BamHI-Hind III fragment including Vspl site was inserted

between the BamHI site and Hind III site of pGT-N28 (5' upstream region of the peptide gene). Then, the HpaI-SalI fragment (3'-downstream region of the peptide gene) was inserted, after modification to XhoI-NotI by linker ligation, between the XhoI and NotI sites to provide pmGFEN28 (Fig. 40).

[Example 36]

Acquisition of homologous recombinant ES cells

Acquisition of homologous recombinant ES cells was carried out using ES cells (RW-4) purchased from Genome Systems in accordance with the attahed manual. Specifically, a 13 kbp (approx.) DNA fragment available upon VspI/NotI digestion of the pmGFEN28 obtained in Example 35 was isolated by agarose gel electrophoresis and, using Bio-Rad Gene Pulser, electroporated into RW-4 cells to obtain recombinant cells. In addition, the same DNA fragment was sent to Genome Systems with a request for electroporation and performed an antibiotic selection of ES cells (MK 2/20). Thus, as shown in Fig. 41, Southern blotting gave the objective homologous recombinant clones No. 92 Hinuma, No. 56FF, and No. 81FF from lane 1, lane 6, and lane 7 where 12 kbp and 5.5 kbp bands could be detected with the 5'-probe and 7.5 kbp and 5.5 kbp bands with the 3'-probe. Using those ES clones, chimera mice can be constructed.

[SEQUENCE LISTING]

INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:82

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

Ala Pro Arg Thr Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu Val Leu
1 5 10 15

Pro Gly Ala Ser Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr
20 25 30

Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val
35 40 45

Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Arg Asp Val Thr Gly Pro
50 55 60

Gly Leu Arg Cys Arg Leu Ser Cys Phe Pro Leu Asp Gly Ser Ala Lys
65 70 75 80

Phe Ser
85

INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:249

(B) TYPE: Nucleic acid

(C) STRANDENESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:2:

GGCACCGAGG ACCTGGCTTC TGTGCTTGCT GCTGCTAGGC TTAGTCCTCC CAGGAGCTTC 60

CAGCCGAGCC CACCAGCACT CCATGGAGAC CCGCACCCCT GACATCAATC CTGCCTGGTA 120

CACGGGTCGT GGGATCAGGC CTGTGGGCCG CTTCGGGAGG AGGAGGGCAG CCCTGAGGGA 180

TGTCACCGGA CCTGGCCTGC GGTGCCGGCT AAGCTGCTTC CCACTGGATG GAAGTGCCAA 240

GTTCTCTCA 249

INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:893

(B) TYPE: Nucleic acid

(C) STRANDENESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: genomic DNA

(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:3:

GGCACCGAGG ACCTGGCTTC TGTGCTTGCT GCTGCTAGGC TTAGTCCTCC CAGGAGCTTC 60

CAGCCGAGCC CACCAGCACT CCATGGAGAC CCGCAGTGAG TGCCTGGCAT ATGGAGGACA 120

GCCACTGTCA CCTCCCATCC ATATGCTTCC CAAATGCCTT GAGTACCCAG CCCCTGAATG 180

GGAGGTTAGC CATCTCCTAA GCCAGTGGTT TCCAACCTTC CTAATACAGA ACTTTTAATA 240

CAGATCCTTA TGTTGTGGTG ACCCCCAGCC AGAAAATTAT TGTGATGCTG TTTTCATAGT 300

TGTAAGTTTT GCTACTGTTA TGGATCATAA TGTTAATATC TGAAATGCAG GATGTCTGAT 360

ATGCGCCCTT CCCCCCAAAC AAAAGGGACA CAACCCACAG GTTGAGAGCC TCTGGGATCT 420

AAGCAAAAGC TACCTTACCA TGCAGTCAGT TGGGAGATTG GTCCTGTTAA GATCTCCCCA 480

GAATGGTCCT GTTTCCTGTC CTCATCATTC CCCTAACCCA TCTTTGTGGG GTCCCTTAAG 540

ACTTTGGAGG ATGACAGTCA GACAGGAAGA GAATACTGAT CCTGGCATAT GTCTAAATAA 600

ATTCCCTAAA GCCACACCAC TGCCCAGATA TGCCCAGCCA GTGTAATCAG GGTGGGTGCC 660

AACATGGCCT GGTGCCCAGG TTTCCATCAG CTTAGGGGCT CCCGTGTCCC ATACGCTGCT 720

CTGACTCTTT CCTTTCCAGC CCCTGACATC AATCCTGCCT GGTACACGGG TCGTGGGATC 780

AGGCCTGTGG GCCGCTTCGG GAGGAGGAGG GCAGCCCTGA GGGATGTCAC CGGACCTGGC 840

CTGCGGTGCC GGCTAAGCTG CTTCCCACTG GATGGAAGTG CCAAGTTCTC TCA 893

INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:31

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:4:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn

1 5 10 15

Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe

20 25 30

INFORMATION FOR SEQ ID NO:5
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:31
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:5:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe
20 25 30

INFORMATION FOR SEQ ID NO:6
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:20
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:6:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe
20 25 30

INFORMATION FOR SEQ ID NO:7
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:9
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:7:

Ser Arg Ala His Gln His Ser Met Glu
1 5 10 15

INFORMATION FOR SEQ ID NO:8

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:9

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:8:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr

1 5

INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:10

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:9:

Gly Arg Gly Ile Arg Pro Val Gly Arg Phe

1 5 10

INFORMATION FOR SEQ ID NO:10

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:91

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:10:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn

1 5 10 15

Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala

20 25 30

Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val

35 40 45

Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr

50 55 60

Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr
65 70 75 80

Val Val Leu Val His Pro Leu Arg Arg Arg Ile
85 90

INFORMATION FOR SEQ ID NO:11

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:59

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:11:

Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu
1 5 10 15

Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly
20 25 30

Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg
35 40 45

Thr Phe Cys Leu Leu Val Val Val Val Val Val
50 55

INFORMATION FOR SEQ ID NO:12

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:370

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:12:

Met Ala Ser Ser Thr Thr Arg Gly Pro Arg Val Ser Asp Leu Phe Ser
1 5 10 15

Gly Leu Pro Pro Ala Val Thr Thr Pro Ala Asn Gln Ser Ala Glu Ala
20 25 30

Ser Ala Gly Asn Gly Ser Val Ala Gly Ala Asp Ala Pro Ala Val Thr
35 40 45

Pro Phe Gln Ser Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val

```
     50                  55                  60
Leu Leu Tyr Ser Val Val Val Val Val Gly Leu Val Gly Asn Cys Leu
 65                  70                  75                  80
Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn
                 85                  90                  95
Phe Leu Ile Gly Asn Leu Ala Leu Ser Asn Val Leu Met Cys Thr Ala
            100                 105                 110
Cys Val Pro Leu Thr Leu Ala Tyr Ala Pro Glu Pro Arg Gly Trp Val
        115                 120                 125
Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr
    130                 135                 140
Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr
145                 150                 155                 160
Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser
                165                 170                 175
Ala Tyr Ala Val Leu Ala Ile Trp Ala Leu Ser Ala Val Leu Ala Leu
            180                 185                 190
Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val
        195                 200                 205
Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu
    210                 215                 220
Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val
225                 230                 235                 240
Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val
                245                 250                 255
Val Pro Gly Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg
            260                 265                 270
Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val Phe Ala
        275                 280                 285
Val Cys Trp Leu Pro Leu His Val Phe Asn Leu Leu Arg Asp Leu Asp
    290                 295                 300
Pro His Ala Ile Asp Pro Tyr Ala Phe Gly Leu Val Gln Leu Leu Cys
305                 310                 315                 320
His Trp Leu Ala Met Ser Ser Ala Cys Tyr Asn Pro Phe Ile Tyr Ala
                325                 330                 335
Trp Leu His Asp Ser Phe Arg Glu Glu Leu Arg Lys Leu Leu Val Ala
```

340 345 350
Trp Pro Arg Lys Ile Ala Pro His Gly Gln Asn Met Thr Val Ser Val
355 360 365
Val Ile
370

INFORMATION FOR SEQ ID NO:13
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:206
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:13:
Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu Tyr Asn Val Thr Asn
1 5 10 15
Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala
20 25 30
Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val
35 40 45
Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Ala Val Thr
50 55 60
Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr
65 70 75 80
Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser
85 90 95
Ala Tyr Ala Val Leu Ala Ile Trp Val Leu Ser Ala Val Leu Ala Leu
100 105 110
Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val
115 120 125
Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu
130 135 140
Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val
145 150 155 160
Ile Leu Leu Ser Tyr Ala Arg Val Ser Val Lys Leu Arg Asn Arg Val
165 170 175
Val Pro Gly Arg Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg

180 185 190
Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val
195 200 205

INFORMATION FOR SEQ ID NO:14
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:126
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:14:
Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser
1 5 10 15
Ala Tyr Ala Val Leu Gly Ile Trp Ala Leu Ser Ala Val Leu Ala Leu
20 25 30
Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val
35 40 45
Ser Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Ile
50 55 60
Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr Leu Leu Pro Leu Leu Ala
65 70 75 80
Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val
85 90 95
Val Pro Gly Ser Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg
100 105 110
Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val
115 120 125

INFORMATION FOR SEQ ID NO:15
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:273
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:15:

CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 60
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180
CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGGTAC 240
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATC 273

INFORMATION FOR SEQ ID NO:16

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:177
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(xi) FEATURE
(C) IDENTIFICATION METHOD: S
(x) SEQUENCE DESCRIPTION; SEQ ID NO:16:

GGCCTGCTGC TGGTCACCTA CCTGCTCCCT CTGCTGGTCA TCCTCCTGTC TTACGTCCGG 60
GTGTCAGTGA AGCTCCGCAA CCGCGTGGTG CCGGGCTGCG TGACCCAGAG CCAGGCCGAC 120
TGGGACCGCG CTCGGCGCCG GCGCACCTTC TGCTTGCTGG TGGTGGTCGT GGTGGTG 177

INFORMATION FOR SEQ ID NO:17

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:1110
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(xi) FEATURE
(C) IDENTIFICATION METHOD: S
(x) SEQUENCE DESCRIPTION; SEQ ID NO:17:

ATGGCCTCAT CGACCACTCG GGGCCCCAGG GTTTCTGACT TATTTTCTGG GCTGCCGCCG 60
GCGGTCACAA CTCCCGCCAA CCAGAGCGCA GAGGCCTCGG CGGGCAACGG GTCGGTGGCT 120
GGCGCGGACG CTCCAGCCGT CACGCCCTTC CAGAGCCTGC AGCTGGTGCA TCAGCTGAAG 180
GGGCTGATCG TGCTGCTCTA CAGCGTCGTG GTGGTCGTGG GGCTGGTGGG CAACTGCCTG 240

CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 300
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 360
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 420
CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGCTAC 480
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 540
CTGGCCATCT GGGCGCTGTC CGCGGTGCTG GCGCTGCCCG CCGCCGTGCA CACCTATCAC 600
GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 660
CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 720
ATCCTCCTGT CTTACGTCCG GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCTGC 780
GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 840
GTGGTGGTCG TGGTGGTGTT CGCCGTCTGC TGGCTGCCGC TGCACGTCTT CAACCTGCTG 900
CGGGACCTCG ACCCCCACGC CATCGACCCT TACGCCTTTG GGCTGGTGCA GCTGCTCTGC 960
CACTGGCTCG CCATGAGTTC GGCCTGCTAC AACCCCTTCA TCTACGCCTG GCTGCACGAC 1020
AGCTTCCGCG AGGAGCTGCG CAAACTGTTG GTCGCTTGGC CCCGCAAGAT AGCCCCCCAT 1080
GGCCAGAATA TGACCGTCAG CGTGGTCATC 1110

INFORMATION FOR SEQ ID NO:18

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:618

(B) TYPE: Nucleic acid

(C) STRANDENESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:18:

CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGTACAACG TGACGAATTT CCTCATCGGC 60
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180
CAGGCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGCTAC 240
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 300
CTGGCCATCT GGGTGCTGTC CGCGGTGCTG GCGCTGCCCG CCGCCGTGCA CACCTATCAC 360
GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 420
CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 480
ATCCTCCTGT CTTACGCCCG GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCCGC 540
GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 600

GTGGTGGTCG TGGTGGTG 618

INFORMATION FOR SEQ ID NO:19

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:378

(B) TYPE: Nucleic acid

(C) STRANDENESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:19:

GTGGTTCTGG TGCACCCGCT ACGTCGGCGC ATTTCACTGA GGCTCAGCGC CTACGCGGTG 60

CTGGGCATCT GGGCTCTATC TGCAGTGCTG GCGCTGCCGG CCGCGGTGCA CACCTACCAT 120

GTGGAGCTCA AGCCCCACGA CGTGAGCCTC TGCGAGGAGT TCTGGGGCTC GCAGGAGCGC 180

CAACGCCAGA TCTACGCCTG GGGGCTGCTT CTGGGCACCT ATTTGCTCCC CCTGCTGGCC 240

ATCCTCCTGT CTTACGTACG GGTGTCAGTG AAGCTGAGGA ACCGCGTGGT GCCTGGCAGC 300

GTGACCCAGA GTCAAGCTGA CTGGGACCGA GCGCGTCGCC GCCGCACTTT CTGTCTGCTG 360

GTGGTGGTGG TGGTAGTG 378

INFORMATION FOR SEQ ID NO:20

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:25

(B) TYPE: Nucleic acid

(C) STRANDENESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:20:

CGTGGSCMTS STGGGCAACN YCCTG 25

INFORMATION FOR SEQ ID NO:21

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:27

(B) TYPE: Nucleic acid

(C) STRANDENESS: Single

(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:21:
GTNGWRRGGC ANCCAGCAGA KGGCAAA 27

INFORMATION FOR SEQ ID NO:22
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:27:
CTGTGYGYSA TYGCNNTKGA YMGSTAC 27

INFORMATION FOR SEQ ID NO:23
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:29
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:23:
AKGWAGWAGG GCAGCCAGCA GANSRYGAA 29

INFORMATION FOR SEQ ID NO:24
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:24
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:24:
CTGACTTATT TTCTGGGCTG CCGC 24

INFORMATION FOR SEQ ID NO:25
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:24
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:25:
AACACCGACA CATAGACGGT GACC 24

INFORMATION FOR SEQ ID NO:26
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:29
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:26:
Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly
20 25

INFORMATION FOR SEQ ID NO:27
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:19
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:27:
Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg

19

INFORMATION FOR SEQ ID NO:28

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:98

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:28:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu
1 5 10 15

Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile
20 25 30

Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg
35 40 45

Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Pro Gly Asp Gly Pro
50 55 60

Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly
65 70 75 80

Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val
85 90 95

Gln Glu

INFORMATION FOR SEQ ID NO:29

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:20

(B) TYPE: Nucleic acid

(C) STRANDENESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:29:

GCICAYCARC AYTGYATGGA 20

INFORMATION FOR SEQ ID NO:30

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:26
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:30:
CCIACGGGIC KDATGCCICK GCCIGC 26

INFORMATION FOR SEQ ID NO:31
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:26
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:31:
ACGGGCCKDA TGCCICKGCC IGCRTA 26

INFORMATION FOR SEQ ID NO:32
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:20
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:32:
CCGGCGTACC AGGCAGGGTT 20

INFORMATION FOR SEQ ID NO:33
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:28
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single

(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:33:
AGGCAGGGTT GATGTCGGGG GTGCGGAT 28

INFORMATION FOR SEQ ID NO:34
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:34:
CTGCCAGCAG AGCCCACCAG CACTCCA 27

INFORMATION FOR SEQ ID NO:35
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:35:
GTGGGGGCCT GGCTCCTCTG CCTGCTG 27

INFORMATION FOR SEQ ID NO:36
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:32
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:36:
GTGTCGACGA ATGAAGGCGG TGGGGGCCTG GC 32

INFORMATION FOR SEQ ID NO:37
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:24
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:37:
AGGCTCCCGC TGTTATTCCT GGAC 24

INFORMATION FOR SEQ ID NO:38
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:98
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:38:

```
Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu
 1               5                  10                  15
Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile
            20                  25                  30
Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg
        35                  40                  45
Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Gly Asp Gly Pro
    50                  55                  60
Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly
65                  70                  75                  80
Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val
                85                  90                  95
Gln Glu
```

INFORMATION FOR SEQ ID NO:39

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:31
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:39:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe
20 25 30

INFORMATION FOR SEQ ID NO:40
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:32
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:40:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30

INFORMATION FOR SEQ ID NO:41
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:33
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(iV) SEQUENCE DESCRIPTION; SEQ ID NO:41:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30
Arg
33

INFORMATION FOR SEQ ID NO:42

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:42:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15

Val Gly Arg Phe
20

INFORMATION FOR SEQ ID NO:43

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:21

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:43:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15

Val Gly Arg Phe Gly
20

INFORMATION FOR SEQ ID NO:44

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH:22

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iV) SEQUENCE DESCRIPTION; SEQ ID NO:44:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15

Val Gly Arg Phe Gly Arg
20

INFORMATION FOR SEQ ID NO:45
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:26
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:45:
CARCAYTCCA TGGAGACAAG AACCCC 26

INFORMATION FOR SEQ ID NO:46
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:24
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:46:
TACCAGGCAG GATTGATACA GGGG 24

INFORMATION FOR SEQ ID NO:47
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:25
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:47:
GGCATCATCC AGGAAGACGG AGCAT 25

INFORMATION FOR SEQ ID NO:48
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:25

(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:48:
AGCAGAGGAG AGGGAGGGTA GAGGA 25

INFORMATION FOR SEQ ID NO:49
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:22
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:49:
ACGTGGCTTC TGTGCTTGCT GC 22

INFORMATION FOR SEQ ID NO:50
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:25
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:50:
GCCTGATCCC GCGGCCCGTG TACCA 25

INFORMATION FOR SEQ ID NO:51
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:26
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:51:
TTGCCCTTCT CCTGCCGAAG CGGCCC 26

INFORMATION FOR SEQ ID NO:52
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:52:
GGCGGGGGCT GCAAGTCGTA CCCATCG 27

INFORMATION FOR SEQ ID NO:53
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:53:
CGGCACTCCA TGGAGATCCG CACCCCT 27

INFORMATION FOR SEQ ID NO:54
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:54:

CAGGCAGGAT TGATGTCAGG GGTGCGG 27

INFORMATION FOR SEQ ID NO:55
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:55:
CATGGAGTGC CGATGGGTAC GACTTGC 27

INFORMATION FOR SEQ ID NO:56
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:56:
GGCCTCCTCG GAGGAGCCAA GGGATGA 27

INFORMATION FOR SEQ ID NO:57
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:27
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:57:
GGGAAAGGAG CCCGAAGGAG AGGAGAG 27

INFORMATION FOR SEQ ID NO:58

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:294
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:58:

ATGAAGGCGG TGGGGGCCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC CCTGCAGGGG 60
GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA CCCCCGACAT CAACCCTGCC 120
TGGTACGCRG GCCGTGGGAT CCGGCCCGTG GGCCGCTTCG GCCGGCGAAG AGCTGCCCYG 180
GGGGACGGAC CCAGGCCTGG CCCCCGGCGT GTGCCGGCCT GCTTCCGCCT GGAAGGCGGY 240
GCTGAGCCCT CCCGAGCCCT CCCGGGGCGG CTGACGGCCC AGCTGGTCCA GGAA 294

INFORMATION FOR SEQ ID NO:59
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:249
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:59:

ATGGCCCTGA AGACGTGGCT TCTGTGCTTG CTGCTGCTAA GCTTGGTCCT CCCAGGGGCT 60
TCCAGCCGAG CCCACCAGCA CTCCATGGAG ACAAGAACCC CTGATATCAA TCCTGCCTGG 120
TACACGGGCC GCGGGATCAG GCCTGTGGGC CGCTTCGGCA GGAGAAGGGC AACCCCGAGG 180
GATGTCACTG GACTTGGCCA ACTCAGCTGC CTCCCACTGG ATGGACGCAC CAAGTTCTCT 240
CAGCGTGGA 249

INFORMATION FOR SEQ ID NO:60
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:261
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double

(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:60:

ATGAAGGTGC TGAGGGCCTG GCTCCTGTGC CTGCTGATGC TGGGCCTGGC CCTGCGGGGA 60
GCTGCAAGTC GTACCCATCG GCACTCCATG GAGATCCGCA CCCCTGACAT CAATCCTGCC 120
TGGTACGCCA GTCGCGGGAT CAGGCCTGTG GGCCGCTTCG GTCGGAGGAG GGCAACCCTG 180
GGGGACGTCC CCAAGCCTGG CCTGCGACCC CGGCTGACCT GCTTCCCCCT GGAAGGCGGT 240
GCTATGTCGT CCCAGGATGG C 261

INFORMATION FOR SEQ ID NO:61
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:30
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:61:

AGATTGGCAT CATCCAGGAA GACGGAGCAT 30

INFORMATION FOR SEQ ID NO:62
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH:31
(B) TYPE: Nucleic acid
(C) STRANDENESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA
(xi) SEQUENCE DESCRIPTION; SEQ ID NO:62:

GTCGACTCAG CAGCACTGTC TTCTCGAGCT G 31

CLAIMS

1. A polypeptide comprising an amino acid sequence represented by SEQ ID NO:1, or a substantial equivalent thereto, or its amide or ester, or a salt thereof.

2. A DNA comprising a DNA having a nucleotide sequence coding for the polypeptide according to claim 1.

3. A DNA according to claim 2, which comprises a nucleotide sequence represented by SEQ ID NO:2 or SEQ ID NO:3.

4. A recombinant vector comprising the DNA according to claim 2.

5. A transformant which is transformed by the DNA according to claim 2 or the recombinant vector according to claim 4.

6. A non-human knock out animal having an inactivated DNA of the DNA according to claim 2.

7. A non-human transgenic animal having the DNA according to claim 2 or its mutein, or the recombinant vector according to claim 4.

8. A non-human animal cell having an inactivated DNA of the DNA according to claim 2.

9. A method for producing the non-human animal cell according to claim 8, which comprises introducing an inactivated DNA of the DNA according to claim 2.

10. A method for producing the polypeptide according to claim 1 or its amide or ester, or the salt thereof, which comprises cultivating the transformant according to claim 5 to produce and accumulate the polypeptide according to claim 1, and collecting the same.

11. A pharmaceutical composition which comprises the polypeptide according to claim 1 or its amide or ester, or the salt thereof.

12. An antibody against the polypeptide according to claim 1 or its amide or ester, or the salt thereof.

Drawings

Fig.1

```
                9          18          27          36          45          54
5' GTG GGC ATG GTG GGC AAC GTC CTG CTG GTG CTG GTG ATC GCG CGG GTG CGC CGG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Val Gly Met Val Gly Asn Val Leu Leu Val Leu Val Ile Ala Arg Val Arg Arg

               63          72          81          90          99         108
   CTG CAC AAC GTG ACG AAC TTC CTC ATC GGC AAC CTG GCC TTG TCC GAC GTG CTC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Leu His Asn Val Thr Asn Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu

               117         126         135         144         153         162
   ATG TGC ACC GCC TGC GTG CCG CTC ACG CTG GCC TAT GCC TTC GAG CCA CGC GGC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Met Cys Thr Ala Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly

               171         180         189         198         207         216
   TGG GTG TTC GGC GGC GGC CTG TGC CAC CTG GTC TTC TTC CTG CAG CCG GTC ACC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Trp Val Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr

               225         234         243         252         261         270
   GTC TAT GTG TCG GTG TTC ACG CTC ACC ACC ATC GCA GTG GAC CGG TAC GTC GTG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr Val Val

               279         288         297
   CTG GTG CAC CCG CTG AGG CGG CGC ATC 3'
   --- --- --- --- --- --- --- --- ---
   Leu Val His Pro Leu Arg Arg Arg Ile
```

Fig. 2

```
                9          18          27          36          45          54
5' GGC CTG CTG CTG GTC ACC TAC CTG CTC CCT CTG CTG GTC ATC CTC CTG TCT TAC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu Ser Tyr

               63          72          81          90          99         108
   GTC CGG GTG TCA GTG AAG CTC CGC AAC CGC GTG GTG CCG GGC TGC GTG ACC CAG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly Cys Val Thr Gln

           117         126         135         144         153         162
   AGC CAG GCC GAC TGG GAC CGC GCT CGG CGC CGG CGC ACC TTC TGC TTG CTG GTG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg Thr Phe Cys Leu Leu Val

           171         180         189         198
   GTG GTC GTG GTG GTG TTT GCC ATC TGC TGG TTG CCT TAC TAC 3'
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Val Val Val Val Val Phe Ala Ile Cys Trp Leu Pro Tyr Tyr
```

Fig. 3

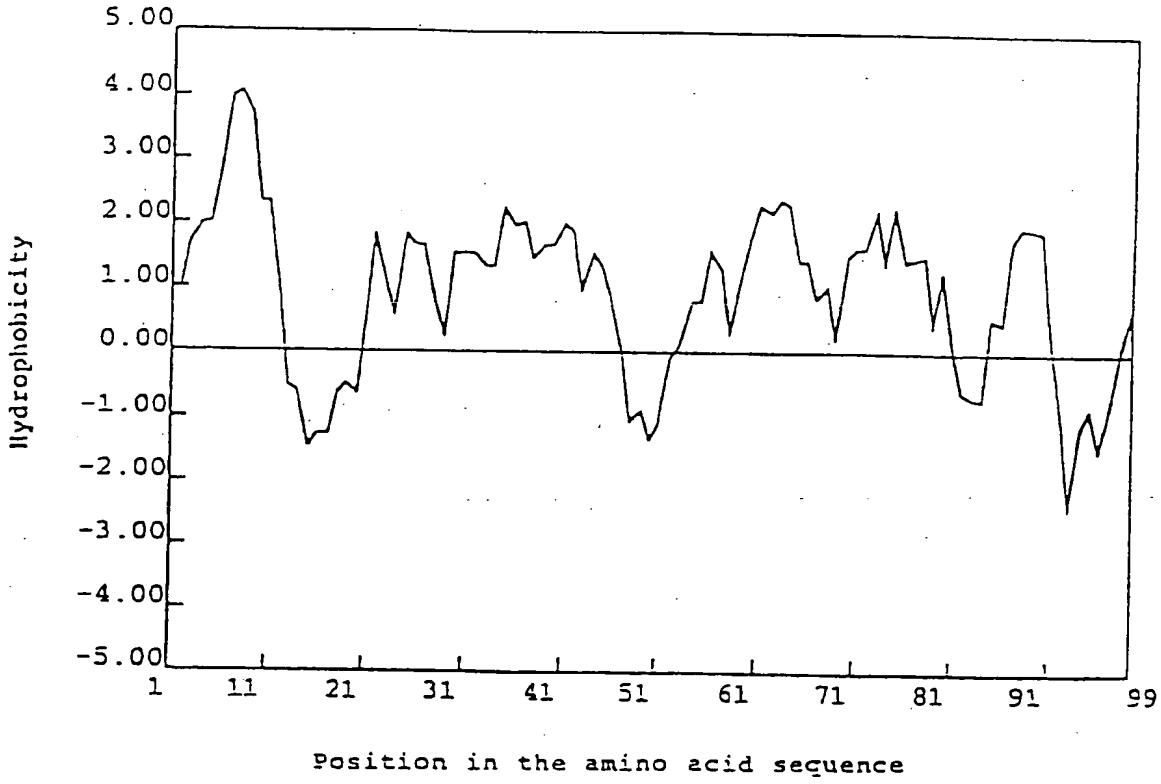


Fig. 4

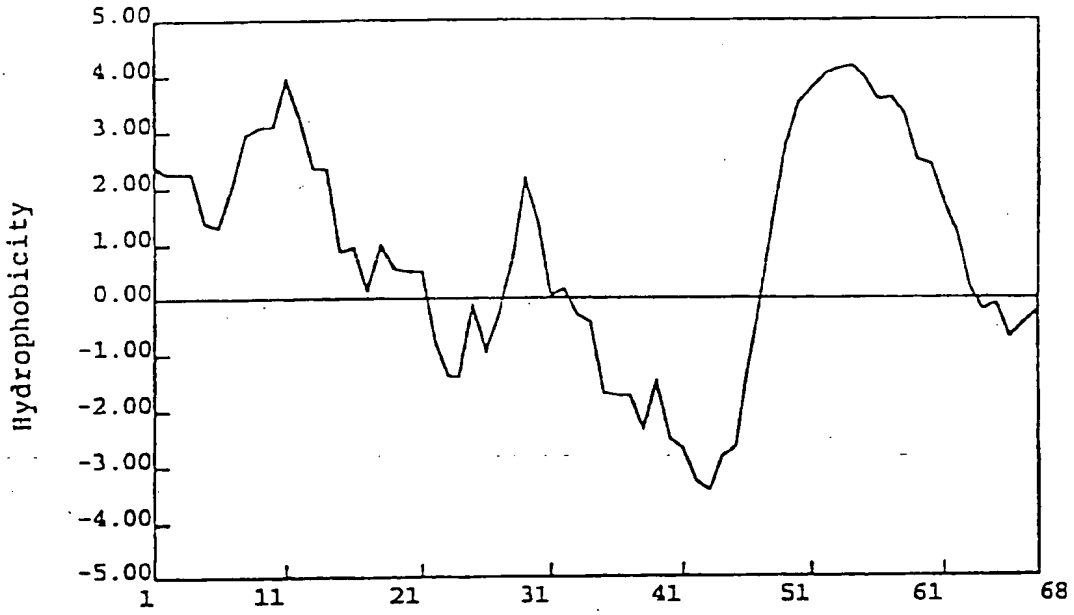


Fig. 5

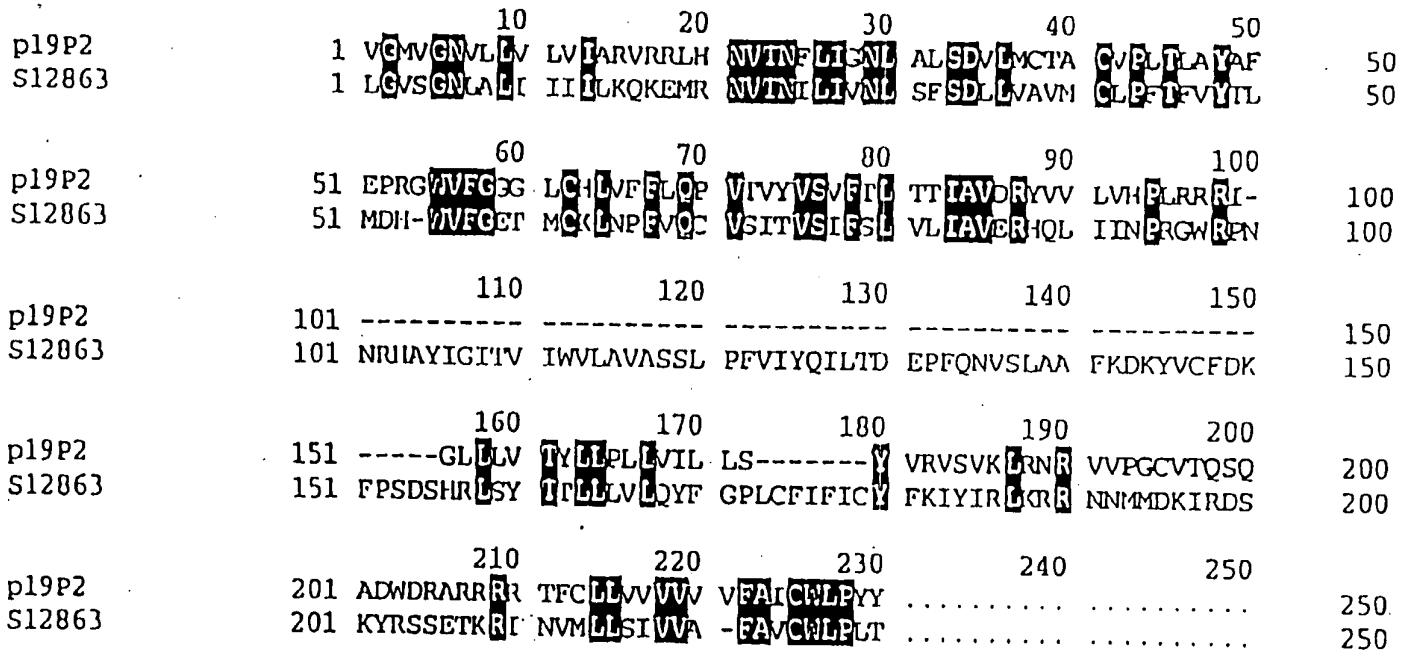


Fig. 6

```
                9          18          27          36          45          54
5'  CTG GGC ATG GTG GGC AAC ATC CTG CTG GTC CTG GTG ATC GCG CGG GTG CGC CGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Gly Met Val Gly Asn Ile Leu Leu Val Leu Val Ile Ala Arg Val Arg Arg

               63          72          81          90          99         108
    CTG TAC AAC GTG ACG AAT TTC CTC ATC GGC AAC CTG GCC TTG TCC GAC GTG CTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Tyr Asn Val Thr Asn Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu

               117         126         135         144         153        162
    ATG TGC ACC GCC TGC GTG CCG CTC ACG CTG GCC TAT GCC TTC GAG CCA CGC GGC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Cys Thr Ala Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly

               171         180         189         198         207        216
    TGG GTG TTC GGC GGC GGC CTG TGC CAC CTG GTC TTC TTC CTG CAG GCG GTC ACC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Trp Val Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Ala Val Thr

               225         234         243         252         261        270
    GTC TAT GTG TCG GTG TTC ACG CTC ACC ACC ATC GCA GTG GAC CGC TAC GTC GTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr Val Val

               279         288         297         306         315        324
    CTG GTG CAC CCG CTG AGG CGG CGC ATC TCG CTG CGC CTC AGC GCC TAC GCT GTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser Ala Tyr Ala Val

               333         342         351         360         369        378
    CTG GCC ATC TGG GTG CTG TCC GCG GTG CTG GCG CTG CCC GCC GCC GTG CAC ACC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Ala Ile Trp Val Leu Ser Ala Val Leu Ala Leu Pro Ala Ala Val His Thr

               387         396         405         414         423        432
    TAT CAC GTG GAG CTC AAG CCG CAC GAC GTG CGC CTC TGC GAG GAG TTC TGG GGC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr His Val Glu Leu Lys Pro His Asp Val Arg Leu Cys Glu Glu Phe Trp Gly

               441         450         459         468         477        486
    TCC CAG GAG CGC CAG CGC CAG CTC TAC GCC TGG GGG CTG CTG CTG GTC ACC TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Gln Glu Arg Gln Arg Gln Leu Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr

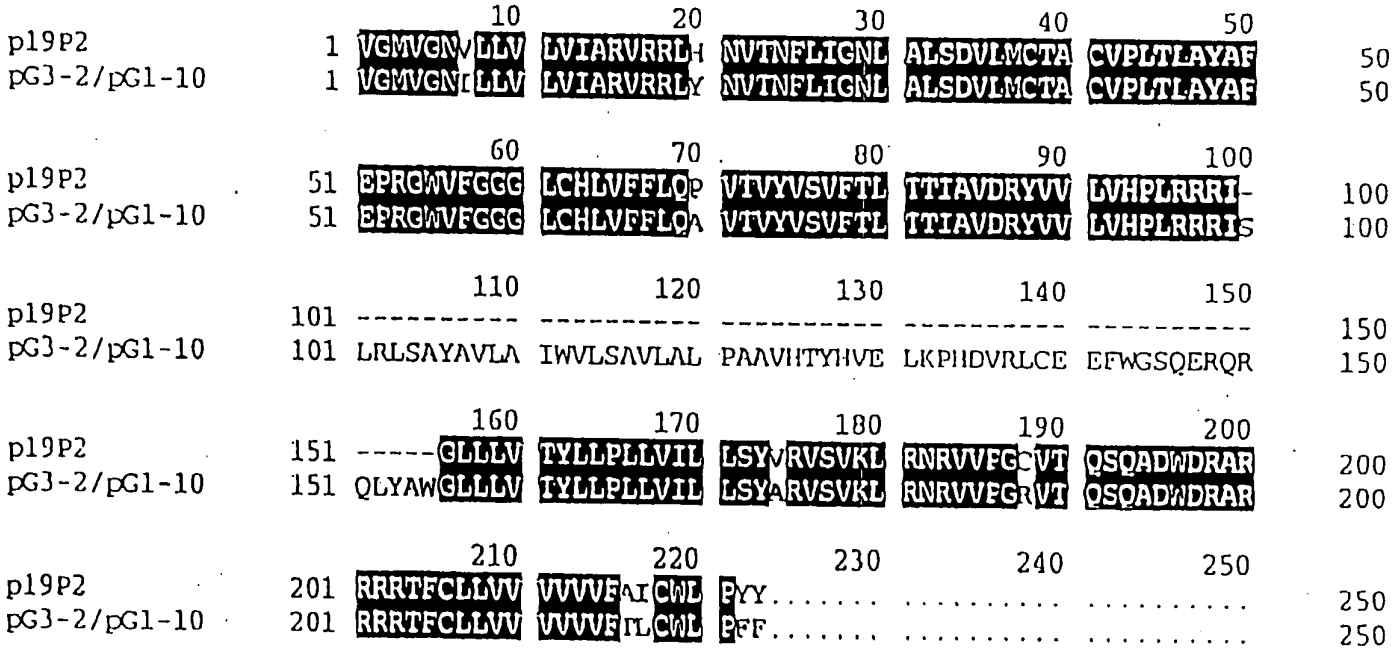
               495         504         513         522         531        540
    CTG CTC CCT CTG CTG GTC ATC CTC CTG TCT TAC GCC CGG GTG TCA GTG AAG CTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Leu Pro Leu Leu Val Ile Leu Leu Ser Tyr Ala Arg Val Ser Val Lys Leu

               549         558         567         576         585        594
    CGC AAC CGC GTG GTG CCG GGC CGC GTG ACC CAG AGC CAG GCC GAC TGG GAC CGC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Arg Asn Arg Val Val Pro Gly Arg Val Thr Gln Ser Gln Ala Asp Trp Asp Arg

               603         612         621         630         639        648
    GCT CGG CGC CGG CGC ACC TTC TGC TTG CTG GTG GTC GTG GTC GTG GTG TTC ACC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ala Arg Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val Phe Thr

               657         666
    CTC TCC TGG CTG CCC TTC TTC 3'
    --- --- --- --- --- --- ---
    Leu Cys Trp Leu Pro Phe Phe
```

Fig. 7



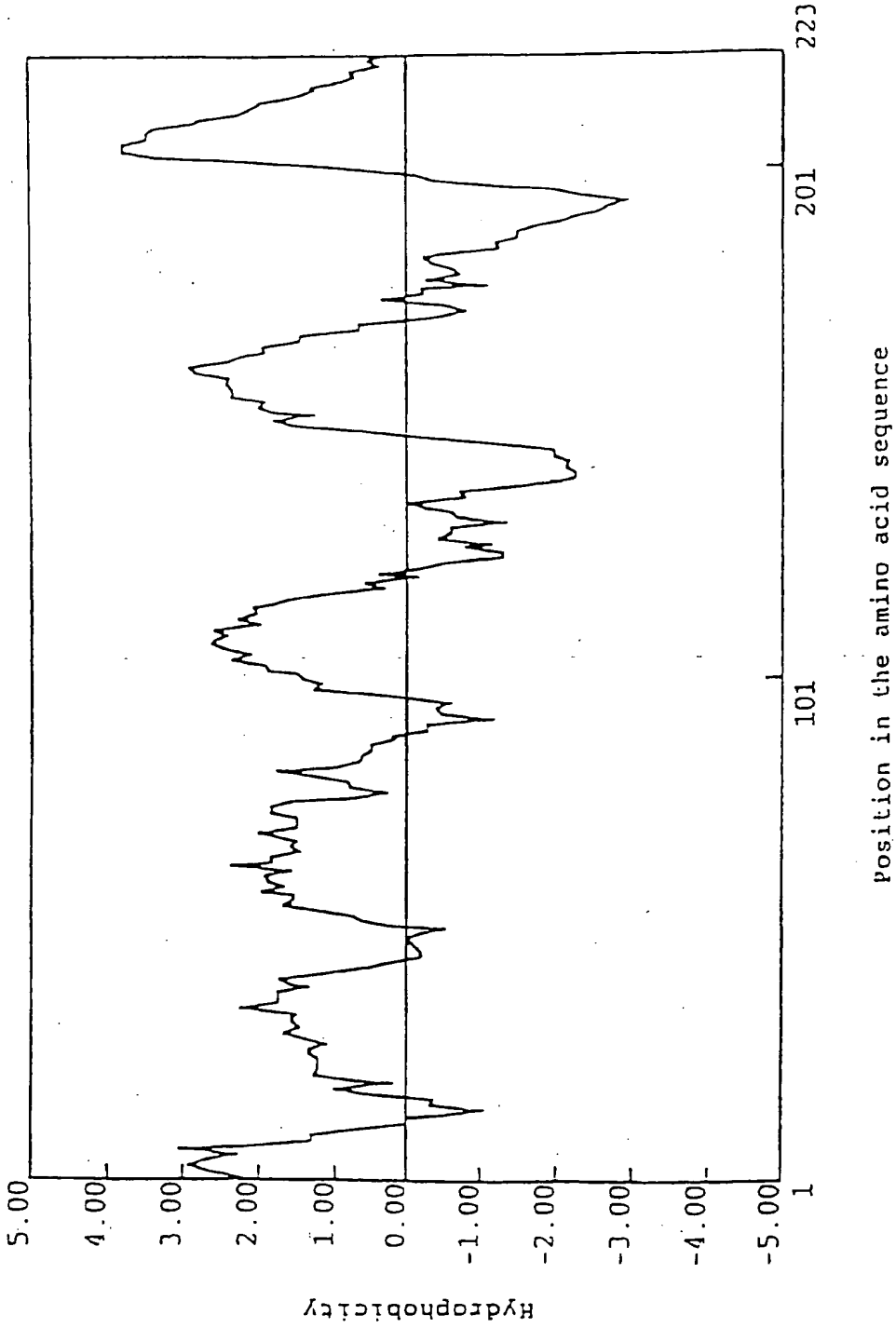


Fig. 8

Fig. 9

```
   1 CATCGTCAAGCAGATGAAGATCATCCACGAGGATGGCTACTCCGACGGCCAGCAGAAATT      60
   1                                                                    1

  61 CTGCCCCTTCTTCCCCGCGAGTGCTTTCCCCGCTCTCCAAACCCCACTCCCAGGTGGCCATG     120
   1                                                           Met      1

 121 GCCTCATCGACCACTCGGGGCCCCAGGGTTTCTGACTTATTTTCTGGGCTGCCGCCGGCG     180
   1 AlaSerSerThrThrArgGlyProArgValSerAspLeuPheSerGlyLeuProProAla      21

 181 GTCACAACTCCCGCCAACCAGAGCGCAGAGGCCTCGGCGGGCAACGGGTCGGTGGCTGGC     240
  21 ValThrThrProAlaAsnGlnSerAlaGluAlaSerAlaGlyAsnGlySerValAlaGly     41

 241 GCGGACGCTCCAGCCGTCACGCCCTTCCAGAGCCTGCAGCTGGTGCATCAGCTGAAGGGG     300
  41 AlaAspAlaProAlaValThrProPheGlnSerLeuGlnLeuValHisGlnLeuLysGly     61

 301 CTGATCGTGCTGCTCTACAGCGTCGTGGTGGTCGTGGGGCTGGTGGGCAACTGCCTGCTG     360
  61 LeuIleValLeuLeuTyrSerValValValValValGlyLeuValGlyAsnCysLeuLeu     81

 361 GTGCTGGTGATCGCCGCGGGTGCGCCGGCTGCACAACGTGACGAACTTCCTCATCGGCAAC     420
  81 ValLeuValIleAlaArgValArgArgLeuHisAsnValThrAsnPheLeuIleGlyAsn     101

 421 CTGGCCTTGTCCGACGTGCTCATGTGCACCGCCTGCGTGCCGCTCACGCTGGCCTATGCC     480
 101 LeuAlaLeuSerAspValLeuMetCysThrAlaCysValProLeuThrLeuAlaTyrAla     121

 481 TTCGAGCCACGGGGCTGGGTGTTCGGCGGGGGCCTGTGCCACCTGGTCTTCTTCCTGCAG     540
 121 PheGluProArgGlyTrpValPheGlyGlyGlyLeuCysHisLeuValPhePheLeuGln     141

 541 CCGGTCACCGTCTATGTGTCGGTGTTCACGCTCACCACCATCGCAGTGGACCGCTACGTC     600
 141 ProValThrValTyrValSerValPheThrLeuThrThrIleAlaValAspArgTyrVal     161

 601 GTGCTGGTGCACCCGCTGAGGCGGCGCATCTCGCTGCGCCTCAGCGCCTACGCTGTGCTG     660
 161 ValLeuValHisProLeuArgArgArgIleSerLeuArgLeuSerAlaTyrAlaValLeu     181

 661 GCCATCTGGGCGCTGTCCGGGGTGCTGGGGCTGCCCGCCGCCGTGCACACCTATCACGTG     720
 181 AlaIleTrpAlaLeuSerAlaValLeuAlaLeuProAlaAlaValHisThrTyrHisVal     201

 721 GAGCTCAAGCCGCACGACGTGCGCCTCTGCGAGGAGTTCTGGGGCTCCCAGGAGCGCCAG     780
 201 GluLeuLysProHisAspValArgLeuCysGluGluPheTrpGlySerGlnGluArgGln     221

 781 CGCCAGCTCTACGCCTGGGGGCTGCTGCTGGTCACCTACCTGCTCCCTCTGCTGGTCATC     840
 221 ArgGlnLeuTyrAlaTrpGlyLeuLeuLeuValThrTyrLeuLeuProLeuLeuValIle     241

 841 CTCCTGTCTTACGTCCGGGTGTCAGTGAAGCTCCGCAACCGCGTGGTGCCGGGCTGCGTG     900
 241 LeuLeuSerTyrValArgValSerValLysLeuArgAsnArgValValProGlyCysVal     261

 901 ACCCAGAGCCAGGCCGACTGGGACCGCGCTCGGCGCCGGCGCACCTTCTGCTTGCTGGTG     960
 261 ThrGlnSerGlnAlaAspTrpAspArgAlaArgArgArgArgThrPheCysLeuLeuVal     281

 961 GTGGTCGTGGTGGTGTTCGCCGTCTGCTGGCTGCCGCTGCACGTCTTCAACCTGCTGCGG    1020
 281 ValValValValValPheAlaValCysTrpLeuProLeuHisValPheAsnLeuLeuArg     301

1021 GACCTCGACCCCCCACGCCATCGACCCTTACGCCTTTGGGCTGGTGCAGCTGCTCTGCCAC    1080
 301 AspLeuAspProHisAlaIleAspProTyrAlaPheGlyLeuValGlnLeuLeuCysHis     321

1081 TGGCTCGCCATGAGTTCGGCCTGCTACAACCCCTTCATCTACGCCTGGCTGCACGACAGC    1140
 321 TrpLeuAlaMetSerSerAlaCysTyrAsnProPheIleTyrAlaTrpLeuHisAspSer     341

1141 TTCCGCGAGGAGCTGCGCAAACTGTTGGTCGCTTGGCCCCGCAAGATAGCCCCCCATGGC    1200
 341 PheArgGluGluLeuArgLysLeuLeuValAlaTrpProArgLysIleAlaProHisGly     361

1201 CAGAATATGACCGTCAGCGTGGTCATCTGATGCCACTTAGCCAGGCCTTGGTCAAGGACC    1260
 361 GlnAsnMetThrValSerValValIle***                                   371

1261 TCCACTTCAACTGGCCTCCTAGGGCACCACTCGAGGTCAATCTGGTGCTTATTCTCAGCA    1320
 371                                                                 371

1321 CCAGAGCTAGC                                                     1331
 371                                                                 371
```

Fig. 10

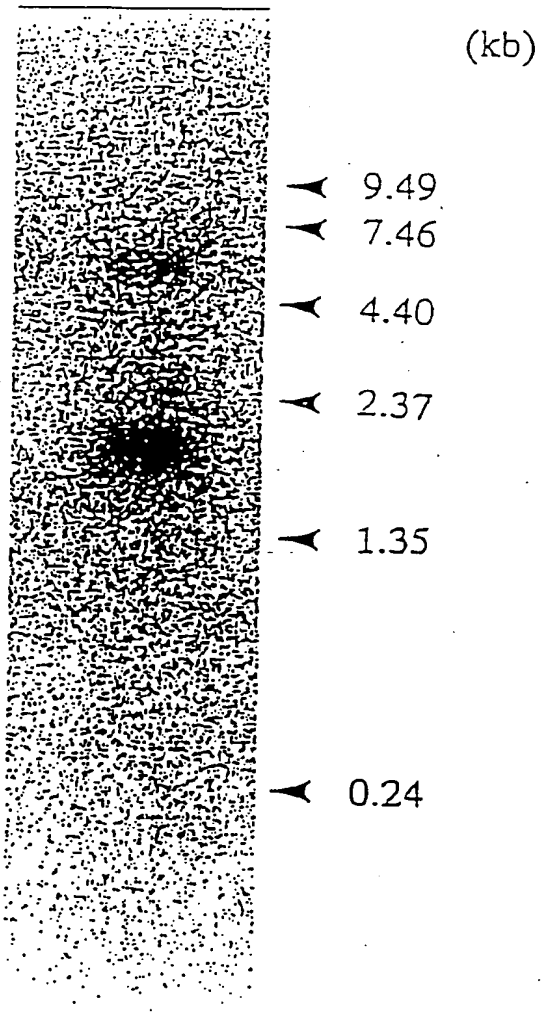


Fig. 11

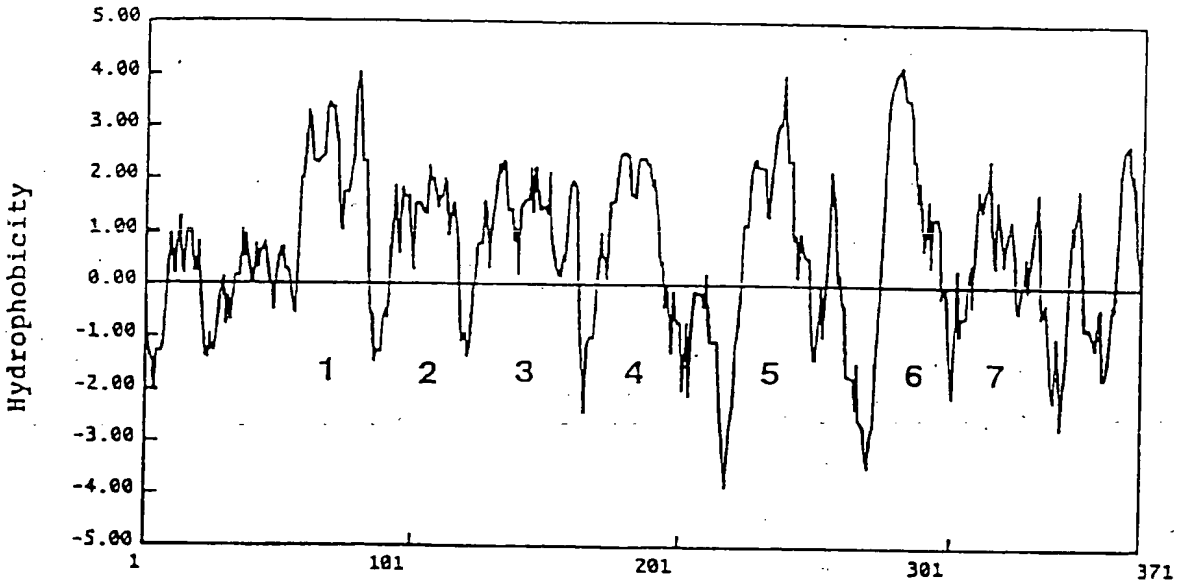


Fig. 12

```
                9          18          27          36          45          54
5'  CTG TGT GTC ATC GCG GTG GAT AGG TAC GTG GTT CTG GTG CAC CCG CTA CGT CGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Cys Val Ile Ala Val Asp Arg Tyr Val Val Leu Val His Pro Leu Arg Arg

               63          72          81          90          99         108
    CGC ATT TCA CTG AGG CTC AGC GCC TAC GCG GTG CTG GGC ATC TGG GCT CTA TCT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Arg Ile Ser Leu Arg Leu Ser Ala Tyr Ala Val Leu Gly Ile Trp Ala Leu Ser

              117         126         135         144         153         162
    GCA GTG CTG GCG CTG CCG GCC GCG GTG CAC ACC TAC CAT GTG GAG CTC AAG CCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ala Val Leu Ala Leu Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro

              171         180         189         198         207         216
    CAC GAC GTG AGC CTC TGC GAG GAG TTC TGG GGC TCG CAG GAG CGC CAA CGC CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    His Asp Val Ser Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln

              225         234         243         252         261         270
    ATC TAC GCC TGG GGG CTG CTT CTG GGC ACC TAT TTG CTC CCC CTG CTG GCC ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ile Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr Leu Leu Pro Leu Leu Ala Ile

              279         288         297         306         315         324
    CTC CTG TCT TAC GTA CGG GTG TCA GTG AAG CTG AGG AAC CGC GTG GTG CCT GGC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly

              333         342         351         360         369         378
    AGC GTG ACC CAG AGT CAA GCT GAC TGG GAC CGA GCG CGT CGC CGC CGC ACT TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg Thr Phe

              387         396         405         414         423         432
    TGT CTG CTG GTG GTG GTG GTG GTA GTG TTC ACG CTC TGC TGG CTG CCC TTC TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Cys Leu Leu Val Val Val Val Val Val Phe Thr Leu Cys Trp Leu Pro Phe Tyr

    CT 3'
    --
    —
```

Fig. 13

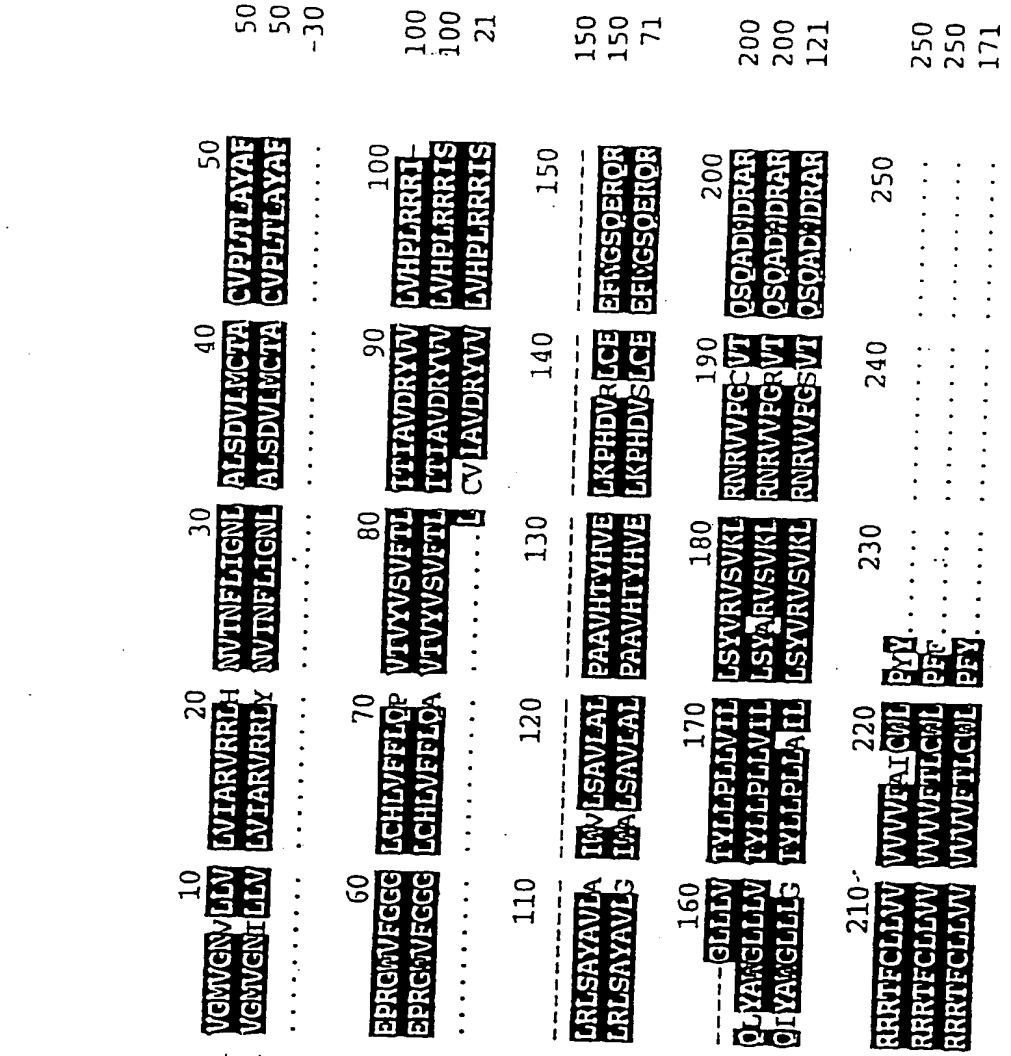


Fig. 14

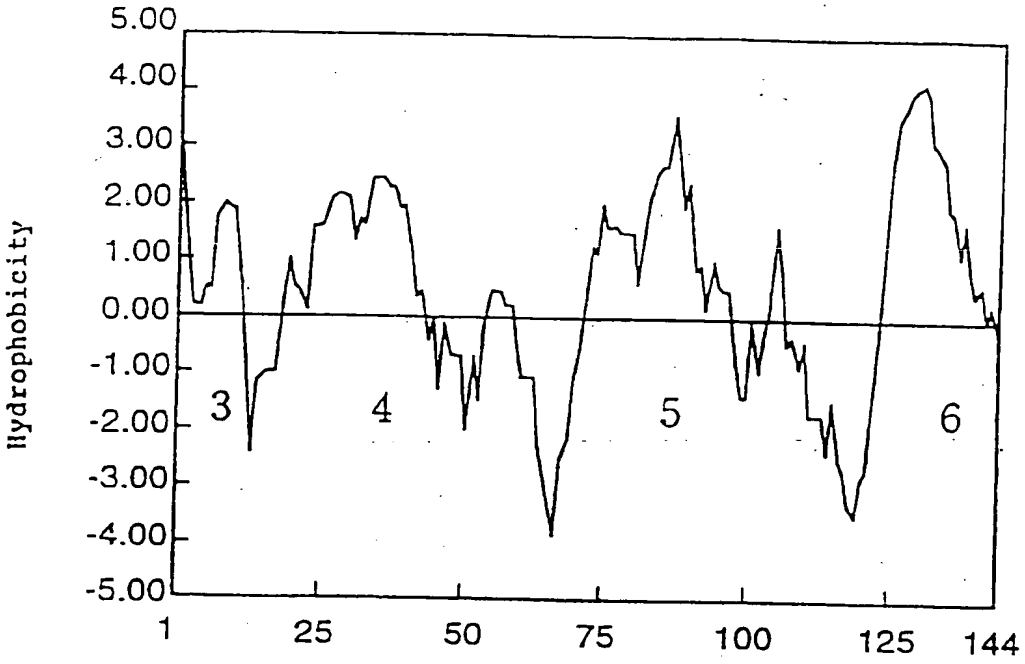


Fig. 15

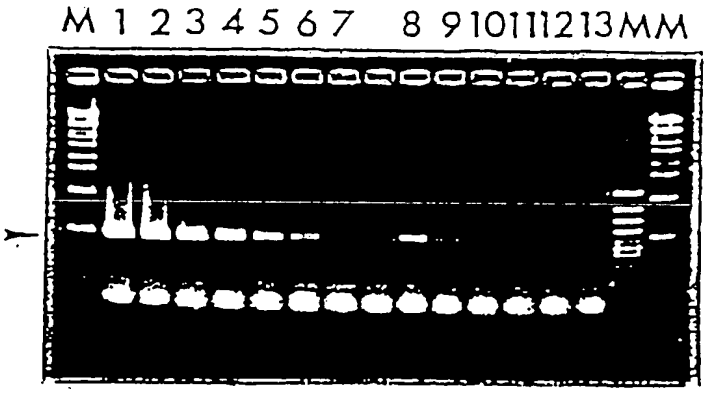
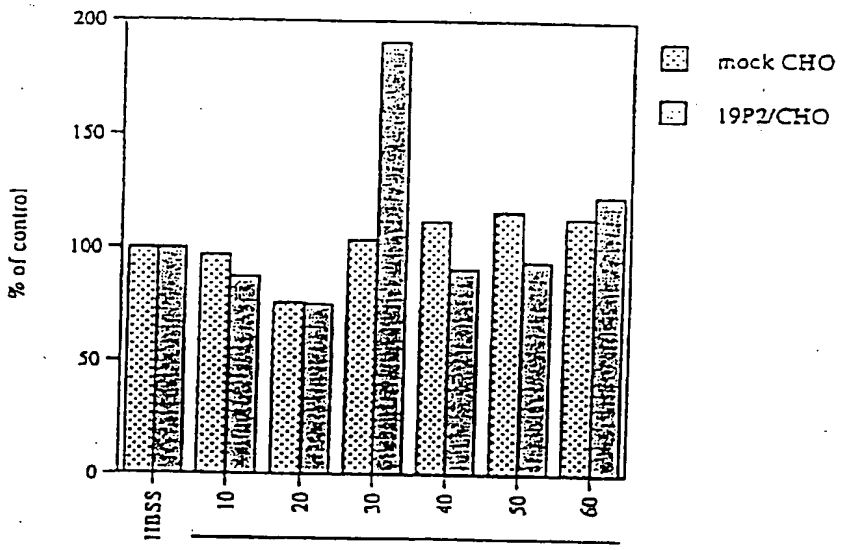


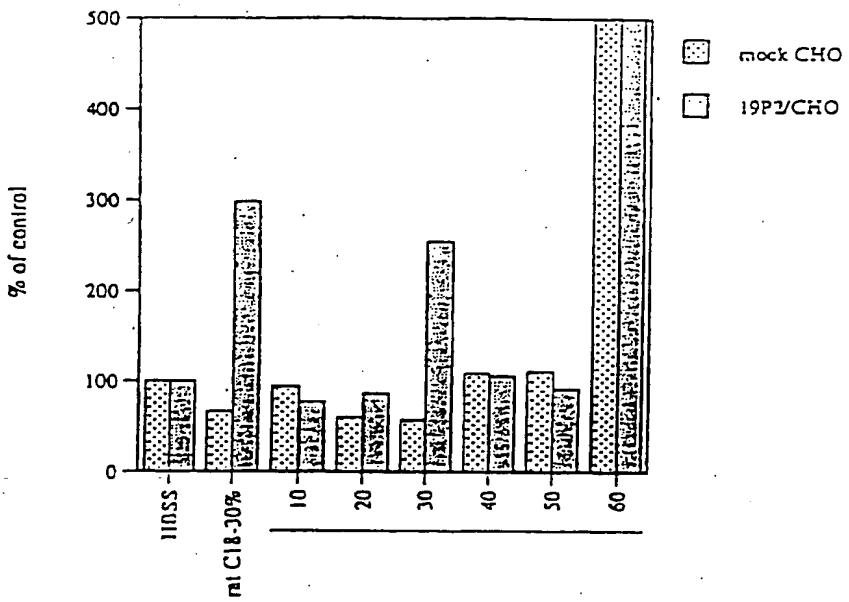
Fig. 16



rat whole brain extract

C_{18}-column CH_3CN elution (%)

Fig. 17



bovine hypothalamus extract

C_{18}-column CH_3CN elution (%)

Fig. 18

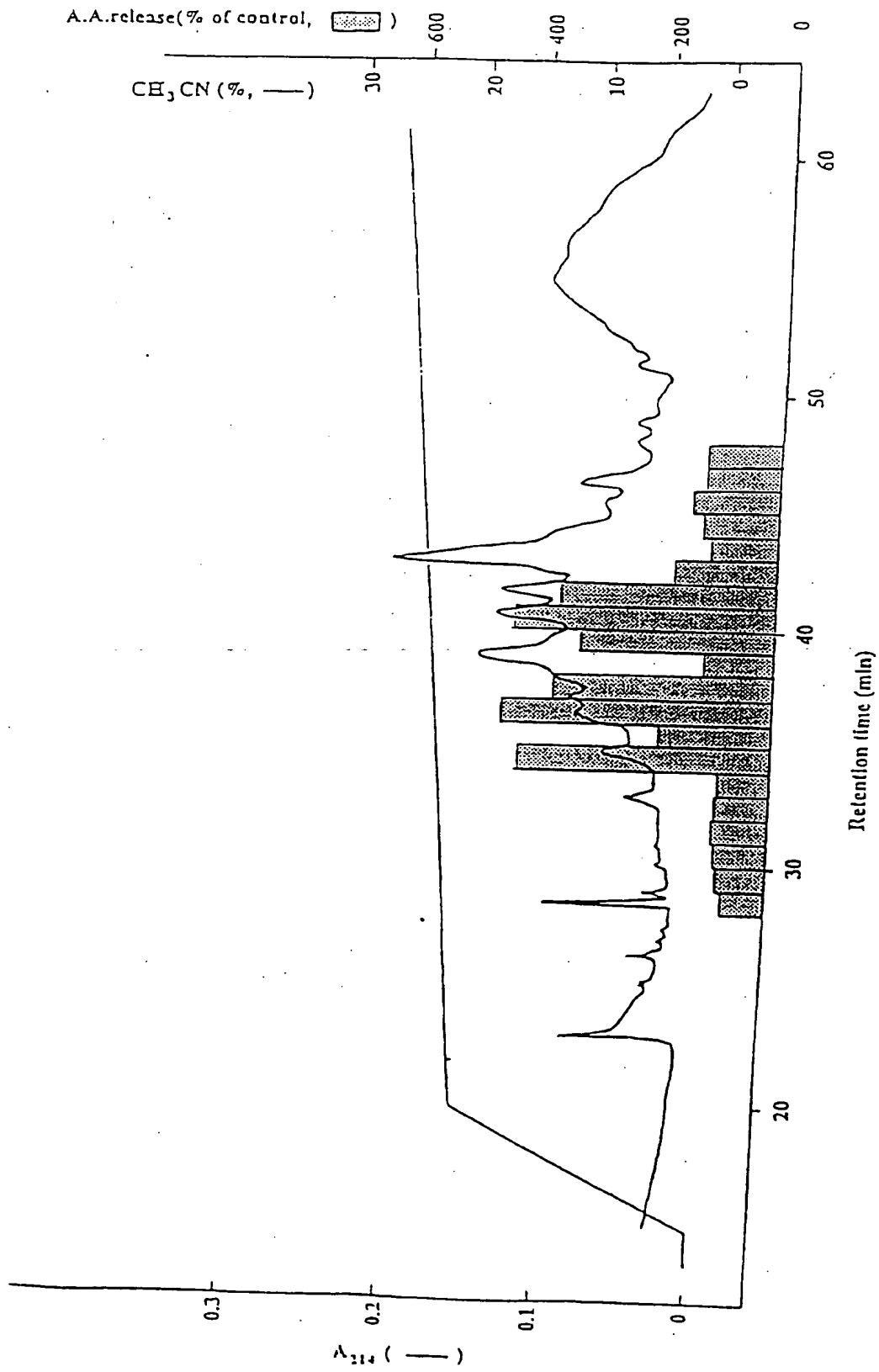


Fig. 19

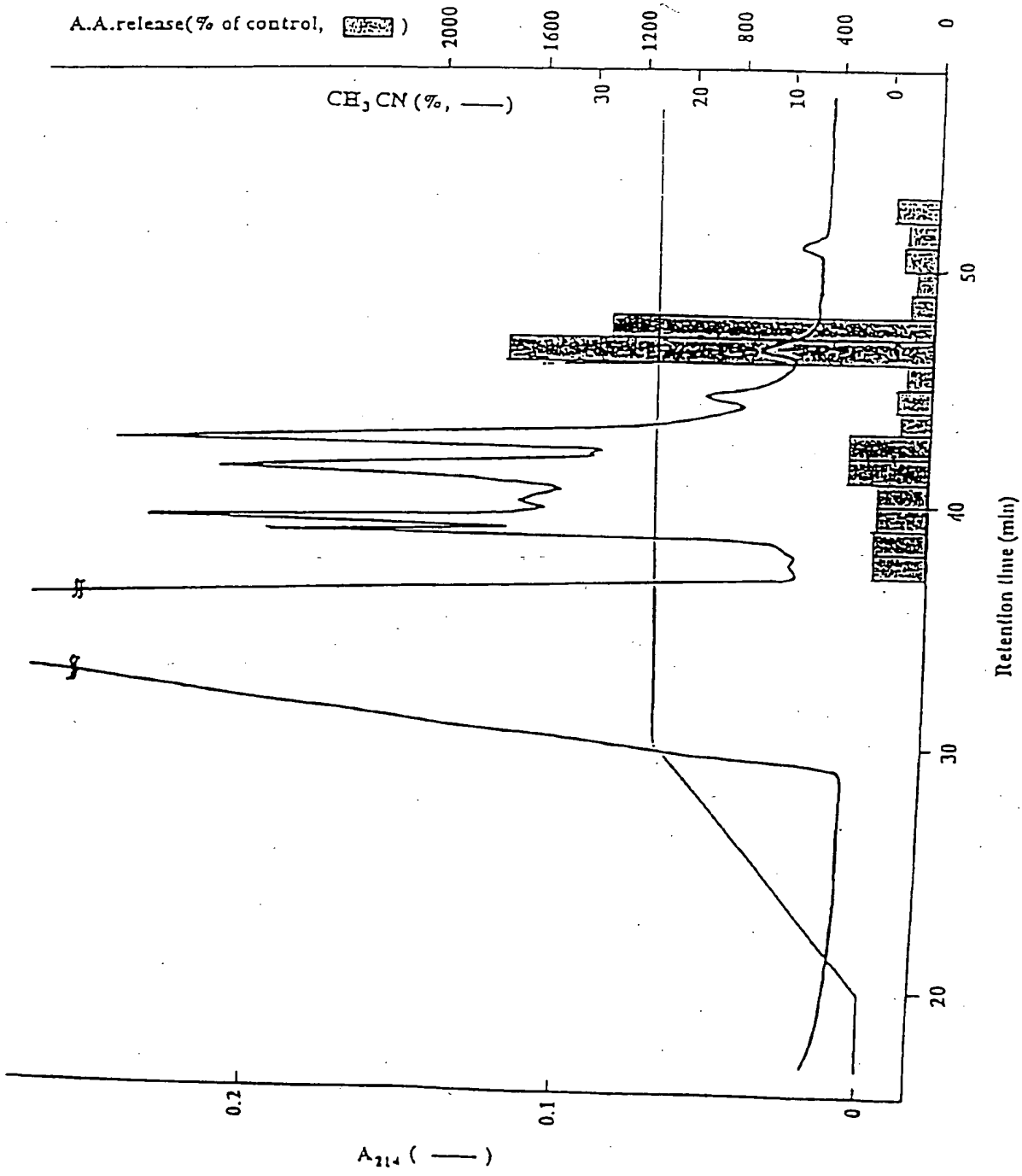


Fig. 20

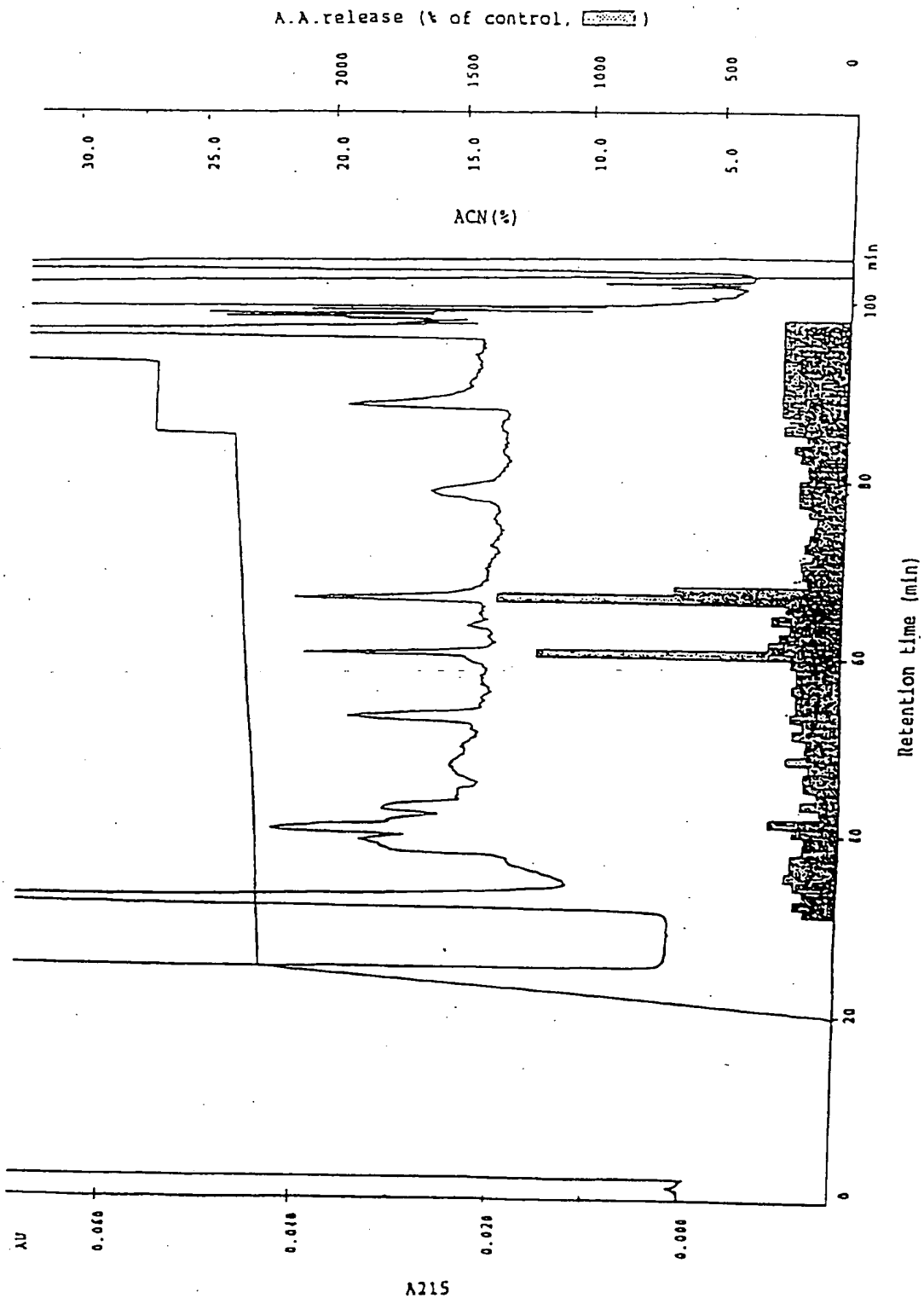


Fig. 21

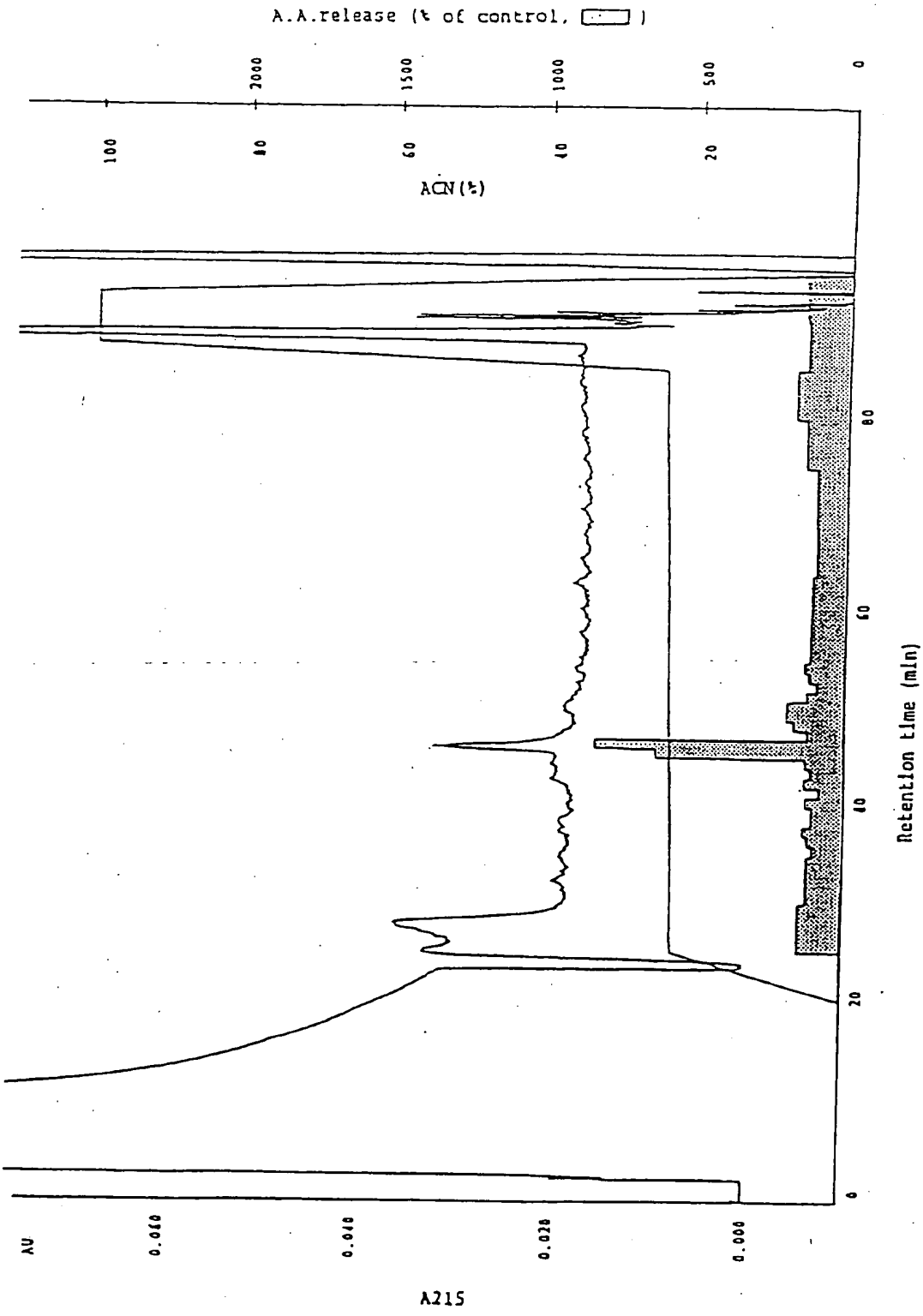


Fig. 22

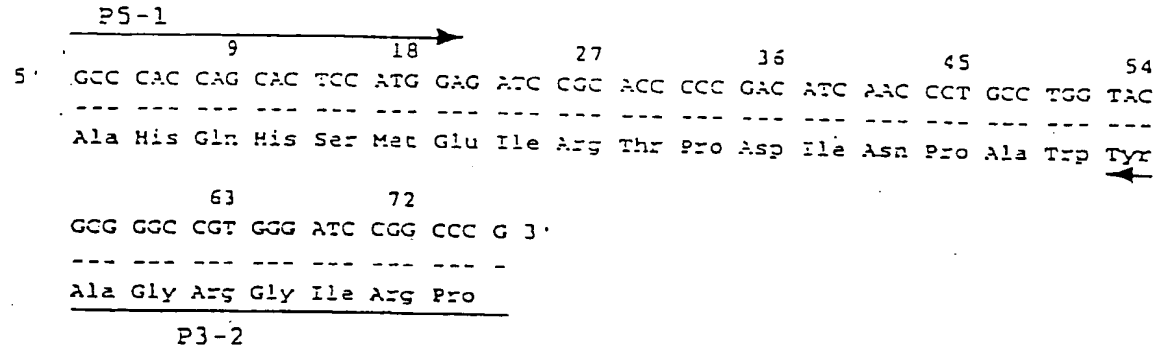


Fig. 23

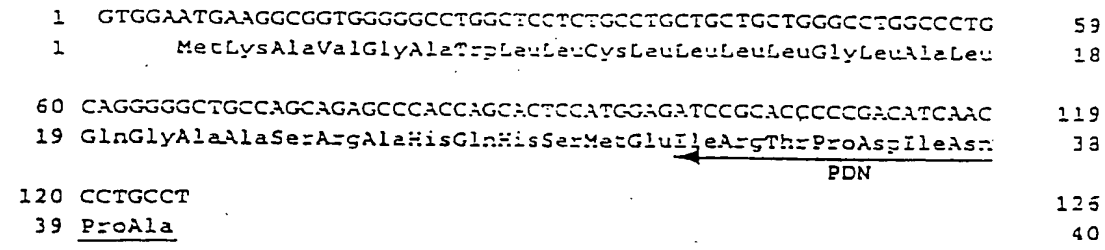


Fig. 24(a)

```
  1  GTGGAATGAAGGCGGTGGGGGCCTGGCTCCTCTGCCTGCTGCTGCTGGGCCTGGCCCTG      59
  1       MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu      18

 60  CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC     119
 19  GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn      38

120  CCTGCCTGGTACGCAGGCCGTGGGATCCGGCCCGTGGGCCGCTTCGGCCGGCGAAGAGCT     179
 39  ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgArgAla      58

180  GCCCCGGGGGACGGACCCAGGCCTGGCCCCCGGCGTGTGCCGGCCTGCTTCCGCCTGGAA     239
 59  AlaProGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu      78

240  GGCGGYGCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCCAGCTGGTCCAGGAA     299
 79  GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu      98

300  TAACAGCGGGAGCCTGCCCCCCACCCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT     359
 98                                                                    98

360  AATAAAAGCAGCTGGCTTGTT                                           380
 98                                                                    98
```

Fig. 24(b)

```
  1  GTGGAATGAAGGCGGTGGGGGCCTGGCTCCTCTGCCTGCTGCTGCTGGGCCTGGCCCTG      59
  1        MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu      18

 60 CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC     119
 19 GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn      38

120 CCTGCCTGGTACGCRGGCCGTGGGATCCGGCCCGTGGGCCGCTTCGGCCGGCGAAGAGCT     179
 39 ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgArgAla      58

180 GCCCTGGGGGACGGACCCAGGCCTGGCCCCCGGCGTGTGCCGGCCTGCTTCCGCCTGGAA     239
 59 AlaLeuGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu      78

240 GGCGGYGCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCCAGCTGGTCCAGGAA     299
 79 GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu      98

300 TAACAGCGGGAGCCTGCCCCCCACCCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT     359
 98                                                                  98

360 AATAAAAGCAGCTGGCTTGTT                                          380
 98                                                                  98
```

Fig. 25

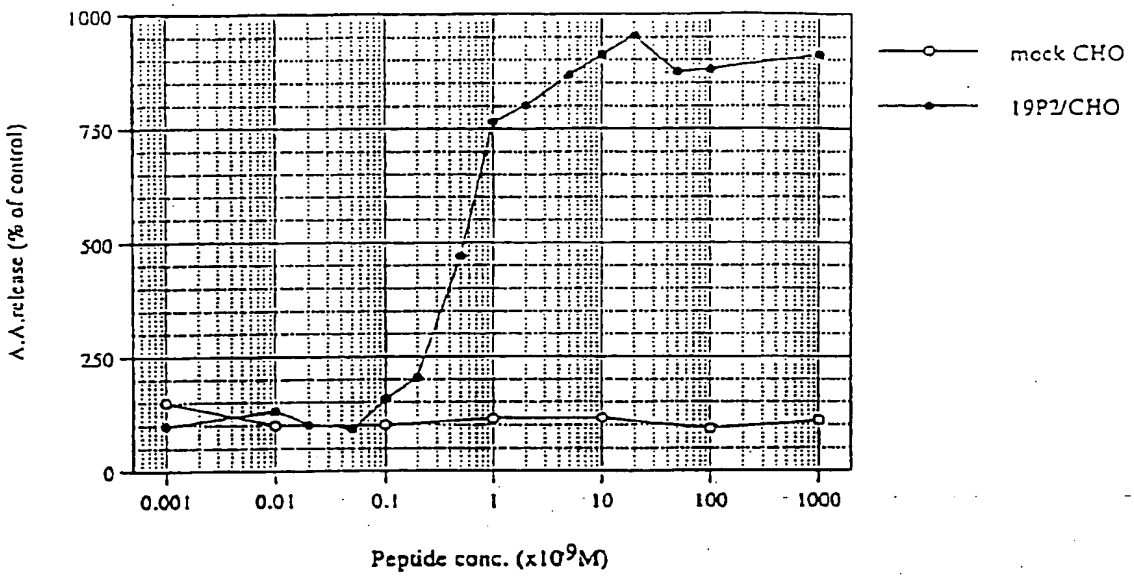


Fig. 26

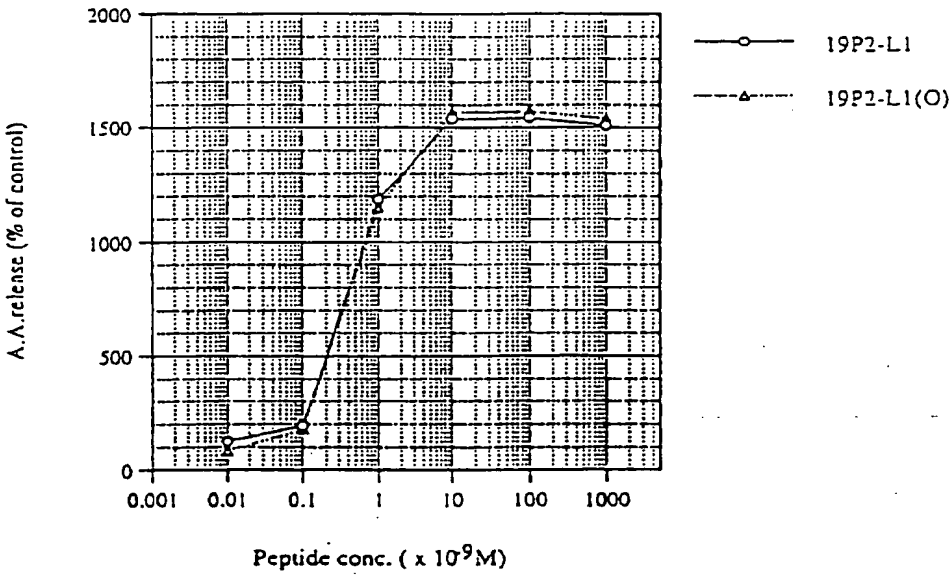


Fig. 27

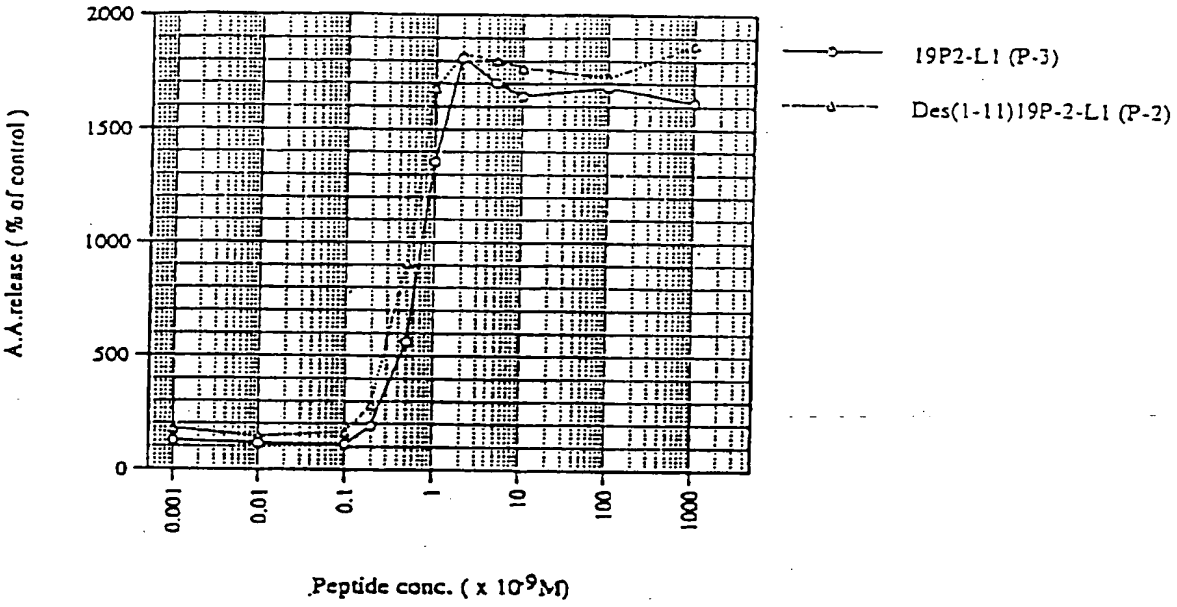


Fig. 28

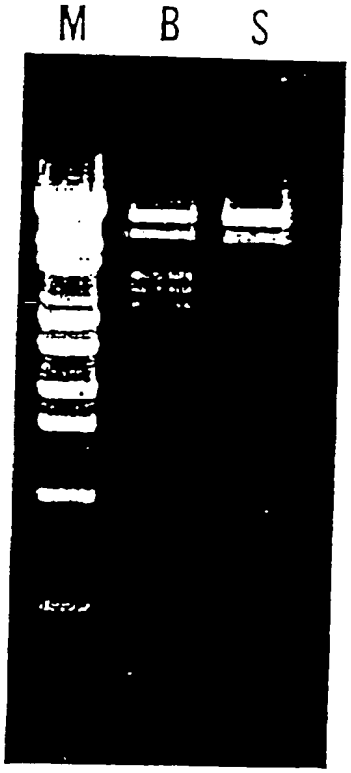


Fig. 29

```
        10          20          30          40          50          60
ATGAAGGCGG TGGGGGCCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC CCTGCAGGGG

        70          80          90         100         110         120
GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA GTGAGTGTCT AGCCCCGCCC

       130         140         150         160         170         180
CTGCCCCCAG GGGTCACAGG GGGGGCCTGG CCACTTCCTG GGCTGGGACA TCCTTGCTAA

       190         200         210         220         230         240
GCATCCTGGG GTTGGGGTTT GGCCTCCTGT TCCCCAGACC CTTCCCCCAG GTGGCCCGGA

       250         260         270         280         290         300
CAGGTGCTCC CAAGGGTCCC GGCCCAGCAC ACGGGGGAGG GTCACTCCTC ACCACACGGG

       310         320         330         340         350         360
TGGCCTGGGG CTGAGTGCAC GTCACCCATG AGAACGGGGC TGTGAGGACA GGAAAGGAAG

       370         380         390         400         410         420
GGGAGTGTGT CCTGGTGTGA GTCTGAAATC CTACTTCCCA AAGCCACCCC AGCACCAGAA

       430         440         450         460         470         480
ATGGGCGCTC CGGGTGAACC TCCTGTGCGG GTGGGTGGTC CTGGCATGGC CTGGGCGACA

       490         500         510         520         530         540
GGCAGCCATG AGCTGAGCAC ACACCCGGCC CGGCCACCAG GGCTGTATGC TCCAGGGCAC

       550         560         570         580         590         600
AGGCCTCCAT GCGCTCTTCT CTCTCTTTCC AGCCCCCGAC ATCAACCCTG CCTGGTACGC

       610         620         630         640         650         660
AGGCCGTGGG ATCCGGCCCG TGGGCCGCTT CGGCCGGCGA AGAGCTGCCC TGGGGGACGG

       670         680         690         700         710         720
ACCCAGGCCT GGCCCCCGGC GTGTGCCGGC CTGCTTCCGC CTGGAAGGCG GTGCTGAGCC

       730         740         750         760         770         780
CTCCCGAGCC CTCCCGGGGC GGCTGACGGC CCAGCTGGTC CAGGAATAA. ..........
```

Fig. 30

```
                      10         20         30         40         50
genome      1 ATGAAGGCGG TGGGGGCCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC      50
cDNA        1 ATGAAGGCGG TGGGGGCCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC      50

                      60         70         80         90        100
genome     51 CCTGCAGGGG GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA     100
cDNA       51 CCTGCAGGGG GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA     100

                     110        120        130        140        150
genome    101 GTGAGTGTCT AGCCCCGCCC CTGCCCCCAG GGGTCACAGG GGGGGCCTGG     150
cDNA      101 ---------- ---------- ---------- ---------- ----------     150

                     160        170        180        190        200
genome    151 CCACTTCCTG GGCTGGGACA TCCTTGCTAA GCATCCTGGG GTTGGGGTTT     200
cDNA      151 ---------- ---------- ---------- ---------- ----------     200

                     210        220        230        240        250
genome    201 GGCCTCCTGT TCCCCAGACC CTTCCCCCAG GTGGCCCGGA CAGGTGCTCC     250
cDNA      201 ---------- ---------- ---------- ---------- ----------     250

                     260        270        280        290        300
genome    251 CAAGGGTCCC GGCCCAGCAC ACGGGGGAGG GTCACTCCTC ACCACACGGG     300
cDNA      251 ---------- ---------- ---------- ---------- ----------     300

                     310        320        330        340        350
genome    301 TGGCCTGGGG CTGAGTGCAC GTCACCCATG AGAACGGGGC TGTGAGGACA     350
cDNA      301 ---------- ---------- ---------- ---------- ----------     350

                     360        370        380        390        400
genome    351 GGAAAGGAAG GGGAGTGTGT CCTGGTGTGA GTCTGAAATC CTACTTCCCA     400
cDNA      351 ---------- ---------- ---------- ---------- ----------     400

                     410        420        430        440        450
genome    401 AAGCCACCCC AGCACCAGAA ATGGGCGCTC CGGGTGAACC TCCTGTGCGG     450
cDNA      401 ---------- ---------- ---------- ---------- ----------     450

                     460        470        480        490        500
genome    451 GTGGGTGGTC CTGGCATGGC CTGGGCGACA GGCAGCCATG AGCTGAGCAC     500
cDNA      451 ---------- ---------- ---------- ---------- ----------     500

                     510        520        530        540        550
genome    501 ACACCGGGCC CGGCCACCAG GGCTGTATGC TCCAGGGCAC AGGCCTCCAT     550
cDNA      501 ---------- ---------- ---------- ---------- ----------     550

                     560        570        580        590        600
genome    551 GCGCTCTTCT CTCTCTTTCC AGCCCCCGAC ATCAACCCTG CCTGGTACGC     600
cDNA      551 ---------- ---------- --CCCCCGAC ATCAACCCTG CCTGGTACGC     600

                     610        620        630        640        650
genome    601 AGGCCGTGGG ATCCGGCCCG TGGGCCGCTT CGGCCGGCGA AGAGCTGCCC     650
cDNA      601 GGGCCGTGGG ATCCGGCCCG TGGGCCGCTT CGGCCGGCGA AGAGCTGCCC     650

                     660        670        680        690        700
genome    651 TGGGGGACGG ACCCAGGCCT GGCCCCCGGC GTGTGCCGGC CTGCTTCCGC     700
cDNA      651 CGGGGGACGG ACCCAGGCCT GGCCCCCGGC GTGTGCCGGC CTGCTTCCGC     700

                     710        720        730        740        750
genome    701 CTGGAAGGCG GTGCTGAGCC CTCCCGAGCC CTCCCGGGGC GGCTGACGGC     750
cDNA      701 CTGGAAGGCG GCGCTGAGCC CTCCCGAGCC CTCCCGGGGC GGCTGACGGC     750

                     760        770        780        790        800
genome    751 CCAGCTGGTC CAGGAATAA. .......... .......... ..........     800
cDNA      751 CCAGCTGGTC CAGGAATAA. .......... .......... ..........     800
```

Fig. 31

```
                9                  18                  27                  36                  45                  54
5' ATG AAG GCG GTG GGG GCC TGG CTC CTC TGC CTG CTG CTG CTG GGC CTG GCC CTG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    M   K   A   V   G   A   W   L   L   C   L   L   L   L   G   L   A   L

               63                  72                  81                  90                  99                 108
   CAG GGG GCT GCC AGC AGA GCC CAC CAG CAC TCC ATG GAG ATC CGC ACC CCC GAC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Q   G   A   A   S   R   A   H   Q   H   S   M   E   I   R   T   P   D

              117                 126                 135                 144                 153                 162
   ATC AAC CCT GCC TGG TAC GCA GGC CGT GGG ATC CGG CCC GTG GGC CGC TTC GGC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    I   N   P   A   W   Y   A   G   R   G   I   R   P   V   G   R   F   G

              171                 180                 189                 198                 207                 216
   CGG CGA AGA GCT GCC CTG GGG GAC GGA CCC AGG CCT GGC CCC CGG CGT GTG CCG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    R   R   R   A   A   L   G   D   G   P   R   P   G   P   R   R   V   P

              225                 234                 243                 252                 261                 270
   GCC TGC TTC CGC CTG GAA GGC GGT GCT GAG CCC TCC CGA GCC CTC CCG GGG CGG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    A   C   F   R   L   E   G   G   A   E   P   S   R   A   L   P   G   R

              279                 288                 297
   CTG ACG GCC CAG CTG GTC CAG GAA TAA 3'
   --- --- --- --- --- --- --- --- ---
    L   T   A   Q   L   V   Q   E   *
```

Fig. 32

```
  1  GGCATCATCCAGGAAGACGGAGCATGGCCCTGAAGACGTGGCTTCTGTGCTTGCTGCTG      59
  1                          MetAlaLeuLysThrTrpLeuLeuCysLeuLeuLeu      12

 60 CTAAGCTTGGTCCTCCCAGGGGCTTCCAGCCGAGCCCACCAGCACTCCATGGAGACAAGA     119
 13 LeuSerLeuValLeuProGlyAlaSerSerArgAlaHisGlnHisSerMetGluThrArg      32

120 ACCCCTGATATCAATCCTGCCTGGTACACGGGCCGCGGGATCAGGCCTGTGGGCCGCTTC     179
 33 ThrProAspIleAsnProAlaTrpTyrThrGlyArgGlyIleArgProValGlyArgPhe      52

180 GGCAGGAGAAGGGCAACCCCGAGGGATGTCACTGGACTTGGCCAACTCAGCTGCCTCCCA     239
 53 GlyArgArgArgAlaThrProArgAspValThrGlyLeuGlyGlnLeuSerCysLeuPro      72

240 CTGGATGGACGCACCAAGTTCTCTCAGCGTGGATAACACCCCAGCTCGAGAAGACAGTGC     299
 73 LeuAspGlyArgThrLysPheSerGlnArgGly***                            83

300 TGCTGAGCCCAAGCCCACACTCCCTGTCCCCTGCAGACCCTCCTCTACCCTCCCTCTCCT     359
 83                                                                 83

360 CTGCT                                                          364
 83                                                                 83
```

Fig. 33

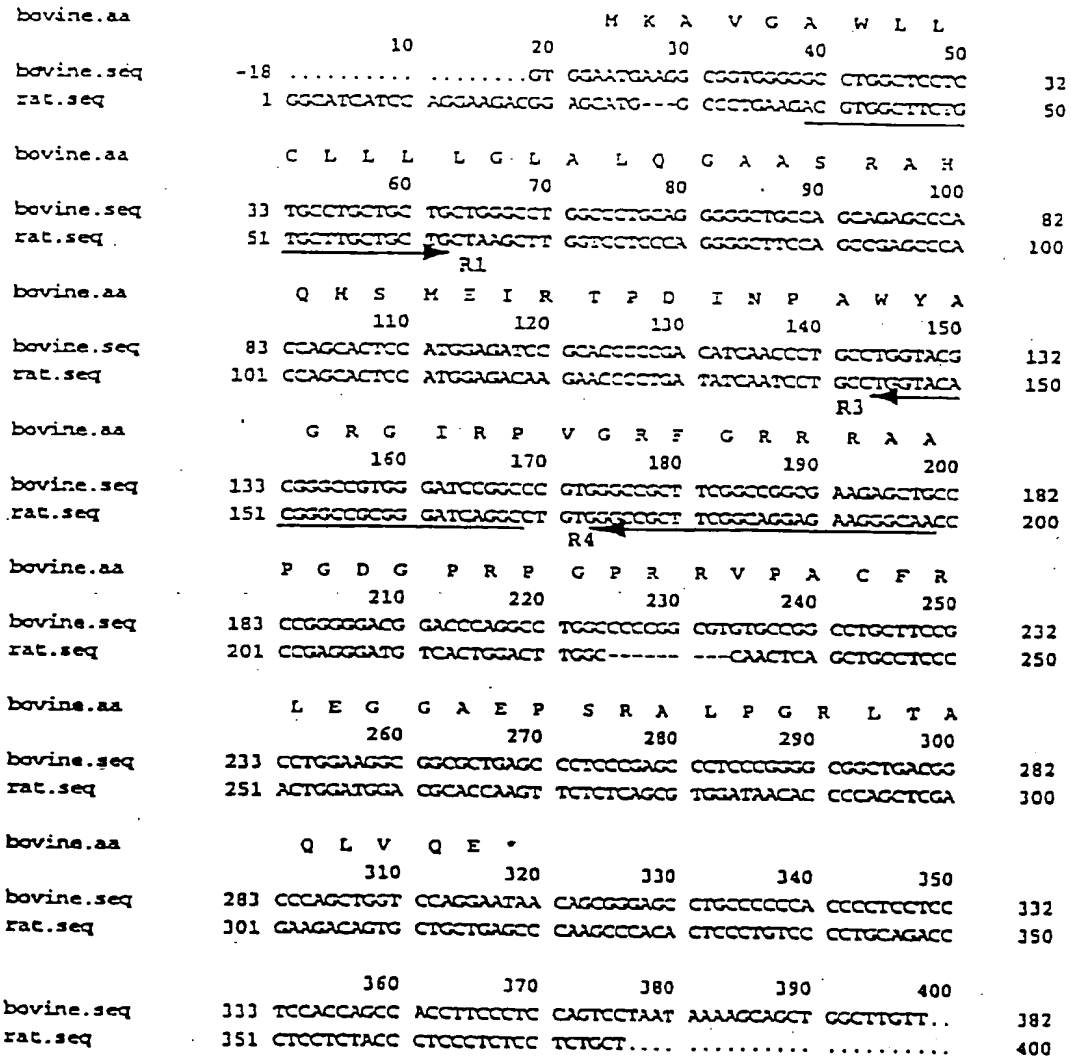


Fig. 34

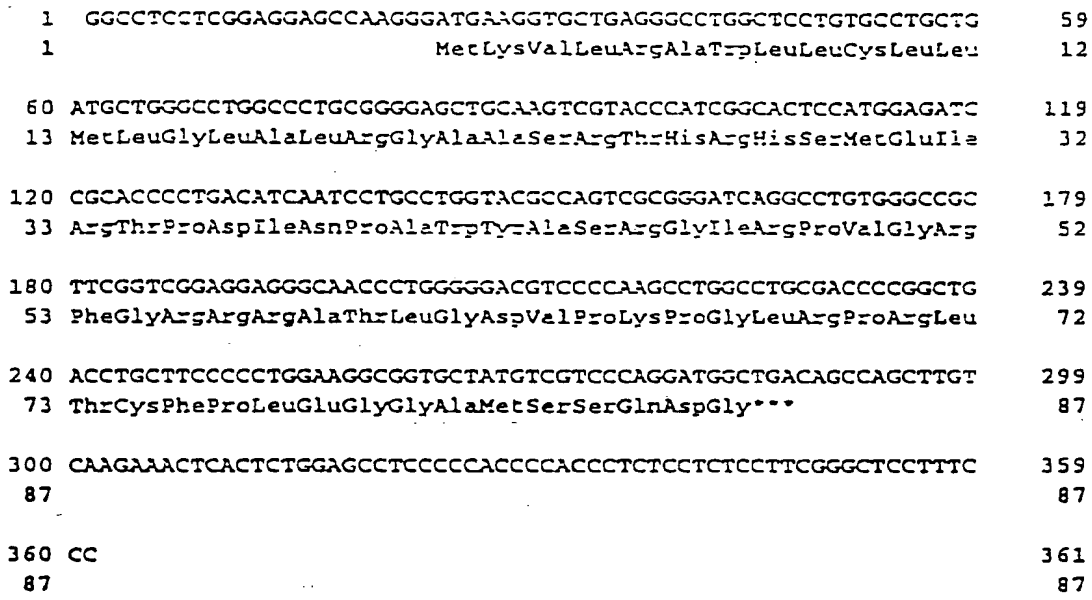


Fig. 35

```
                        10         20         30         40         50
bovine.aa          1 MKAVGAWLLC LLLLGLALQG AASRAHQHSM EIRTPDINPA WYAGRGIRPV  50
rat.aa             1 M-ALKTWLLC LLLLSLVLPG ASSRAHQHSM ETRTPDINPA WYTGRGIRPV  50
human.aa           1 MKVLRAWLLC LLMLGLALRG AASRTHEHSM EIRTPDINPA WYASRGIRPV  50

                        60         70         80         90        100
bovine.aa         51 GRFGRRRAAP GDGPRPGPPR VPACPRLEGG AEPSRALPGR LTAQLVQE*.  100
rat.aa            51 GRFGRRRATP RDVTGLG--- QLSCLPLDGR TKFSQRG*.. ..........  100
human.aa          51 GRFGRRRATL GDVPKPGLRP RLTCFPLEGG AMSSQDG*.. ..........  100
```

Fig. 36

```
        10          20          30          40          50          60
AGATCTGGCA TCATCCAGGA AGACGGAGCA TGGCACCGAG GACCTGGCTT CTGTGCTTGC

        70          80          90         100         110         120
TGCTGCTAGG CTTAGTCCTC CCAGGAGCTT CCAGCCGAGC CCACCAGCAC TCCATGGAGA

       130         140         150         160         170         180
CCCGCAGTGA GTGCCTGGCA TATGGAGGAC AGCCACTGTC ACCTCCCATC CATATGCTTC

       190         200         210         220         230         240
CCAAATGCCT TGAGTACCCA GCCCCTGAAT GGGAGGTTAG CCATCTCCTA AGCCAGTGGT

       250         260         270         280         290         300
TTCCAACCTT CCTAATACAG AACTTTTAAT ACAGATCCTT ATGTTGTGGT GACCCCCAGC

       310         320         330         340         350         360
CAGAAAATTA TTGTGATGCT GTTTTCATAG TTGTAAGTTT TGCTACTGTT ATGGATCATA

       370         380         390         400         410         420
ATGTTAATAT CTGAAATGCA GGATGTCTGA TATGCGCCCT TCCCCCCAAA CAAAAGGGAC

       430         440         450         460         470         480
ACAACCCACA GGTTGAGAGC CTCTGGGATC TAAGCAAAAG CTACCTTACC ATGCAGTCAG

       490         500         510         520         530         540
TTGGGAGATT GGTCCTGTTA AGATCTCCCC AGAATGGTCC TGTTTCCTGT CCTCATCATT

       550         560         570         580         590         600
CCCCTAACCC ATCTTTGTGG GGTCCCTTAA GACTTTGGAG GATGACAGTC AGACAGGAAG

       610         620         630         640         650         660
AGAATACTGA TCCTGGCATA TGTCTAAATA AATTCCCTAA AGCCACACCA CTGCCCAGAT

       670         680         690         700         710         720
ATGCCCAGCC AGTGTAATCA GGGTGGGTGC CAACATGGCC TGGTGCCCAG GTTTCCATCA

       730         740         750         760         770         780
GCTTAGGGGC TCCCGTGTCC CATACGCTGC TCTGACTCTT TCCTTTCCAG CCCCTGACAT

       790         800         810         820         830         840
CAATCCTGCC TGGTACACGG GTCGTGGGAT CAGGCCTGTG GGCCGCTTCG GGAGGAGGAG

       850         860         870         880         890         900
GGCAGCCCTG AGGGATGTCA CCGGACCTGG CCTGCGGTGC CGGCTAAGCT GCTTCCCACT

       910         920         930         940         950         960
GGATGGAAGT GCCAAGTTCT CTCACAGCTC GAGAAGACAG TGCTGCTGAG TCGAC.....
```

Fig. 37

File Name : mouse Genome19P2L

AG ATC TGG CAT CAT CCA GGA AGA CGG AGC ATG GCA CCG AGG ACC TGG CTT CTG TGC
Met Ala Pro Arg Thr Trp Leu Leu Cys

TTG CTG CTG CTA GGC TTA GTC CTC CCA GGA GCT TCC AGC CGA GCC CAC CAG CAC
Leu Leu Leu Leu Gly Leu Val Leu Pro Gly Ala Ser Ser Arg Ala His Gln His

TCC ATG GAG ACC CGC A GT GAG TGC CTG GCA TAT GGA GGA CAG CCA CTG TCA CCT
Ser Met Glu Thr Arg

CCC ATC CAT ATG CTT CCC AAA TGC CTT GAG TAC CCA GCC CCT GAA TGG GAG GTT

AGC CAT CTC CTA AGC CAG TGG TTT CCA ACC TTC CTA ATA CAG AAC TTT TAA TAC

AGA TCC TTA TGT TGT GGT GAC CCC CAG CCA GAA AAT TAT TGT GAT GCT GTT TTC

ATA GTT GTA AGT TTT GCT ACT GTT ATG GAT CAT AAT GTT AAT ATC TGA AAT GCA

GGA TGT CTG ATA TGC GCC CTT CCC CCC AAA CAA AAG GGA CAC AAC CCA CAG GTT

GAG AGC CTC TGG GAT CTA AGC AAA AGC TAC CTT ACC ATG CAG TCA GTT GGG AGA

TTG GTC CTG TTA AGA TCT CCC CAG AAT GGT CCT GTT TCC TGT CCT CAT CAT TCC

CCT AAC CCA TCT TTG TGG GGT CCC TTA AGA CTT TGG AGG ATG ACA GTC AGA CAG

GAA GAG AAT ACT GAT CCT GGC ATA TGT CTA AAT AAA TTC CCT AAA GCC ACA CCA

CTG CCC AGA TAT GCC CAG CCA GTG TAA TCA GGG TGG GTG CCA ACA TGG CCT GGT

GCC CAG GTT TCC ATC AGC TTA GGG GCT CCC GTG TCC CAT ACG CTG CTC TGA CTC

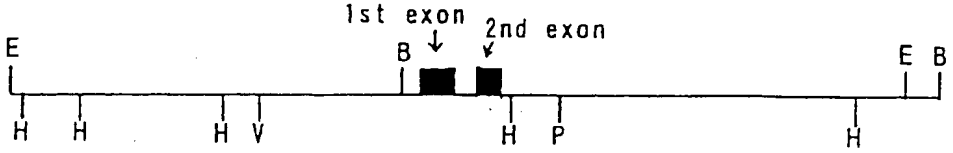
TTT CCT TTC CAG CC CCT GAC ATC AAT CCT GCC TGG TAC ACG GGT CGT GGG ATC
Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile

AGG CCT GTG GGC CGC TTC GGG AGG AGG ACG GCA GCC CTG AGG GAT GTC ACC GGA
Arg Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Arg Asp Val Thr Gly

CCT GGC CTG CGG TGC CGG CTA AGC TGC TTC CCA CTG GAT GGA AGT GCC AAG TTC
Pro Gly Leu Arg Cys Arg Leu Ser Cys Phe Pro Leu Asp Gly Ser Ala Lys Phe

TCT CAC AGC TCG AGA AGA CAG TGC TGC TGA GTC GAC
Ser His Ser Ser Arg Arg Gln Cys Cys •••

Fig. 38



B : Bam HI
E : Eco RI
H : Hind III
V : Vsp I
P : Hpa I

Fig. 39

```
                9          18          27          36          45          54
5'  TGA CCA CAA GGC TGC CCA TAA ATT TGC TTC CCA CCA AGG CCT GGG TGT CTA CTT
               63          72          81          90          99         108
    CCC AGC TTT TGA CAC AGA TGG ACA GAC AGA CCC AAG GAT GTC CCA AGA CAG CCA
              117         126         135         144         153         162
    CCT GTG CAC AGC TCA CAC CTC TCC CCT GAT AAT TGT AGC TAT GTG CAC TAA ACA
              171         180         189         198         207         216
    TAT GCA TTT GCA CAC CTT ATA GGC ACG GAC ACG CAC CAC ACA CAC AAG TAC ATT
              225         234         243         252         261         270
    TGT ACA AAC AAC CTA GGG TCC CTT CTG GCT TTG TGC ATA CAA CGT ACT TTG CAT
              279         288         297         306         315         324
    TTT TAC CCC CAG GCT TCA GGA TCC AAT TTT CAG GGC ATC ATT CAG GAA GCC GGA

              333         342         351         360         369         378
    AGC ATG GCA CCG AGG ACC TGG CTT CTG TGC TTG CTG CTG CTA GGC TTA GTC CTC
        Met Ala Pro Arg Thr Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu Val Leu

              387         396         405         414         423         432
    CCA GGA GCT TCC AGC CGA GCC CAC CAG CAC TCC ATG GAG ACC CGC A GT GAG TGC
    Pro Gly Ala Ser Ser Arg Ala His Gln His Ser Met Glu Thr Arg

              441         450         459         468         477         486
    CTG GCA TAT GGA GGA CAG CCA CTG TCA CCT CCC ATC CAT ATG CTT CCC AAA TGC
              495         504         513         522         531         540
    CTT GAG TAC CCA GCC CCT GAA TGG GAG GTT AGC CAT CTC CTA AGC CAG TGG TTT
              549         558         567         576         585         594
    CCA ACC TTC CTA ATA CAG AAC TTT TAA TAC AGA TCC TTA TGT TGT GGT GAC CCC
              603         612         621         630         639         648
    CAG CCA GAA AAT TAT TGT GAT GCT GTT TTC ATA GTT GTA AGT TTT GCT ACT GTT
              657         666         675         684         693         702
    ATG GAT CAT AAT GTT AAT ATC TGA AAT GCA GGA TGT CTG ATA TGC GCC CTT CCC
              711         720         729         738         747         756
    CCC AAA CAA AAG GGA CAC AAC CCA CAG GTT GAG AGC CTC TGG GAT CTA AGC AAA
              765         774         783         792         801         810
    AGC TAC CTT ACC ATG CAG TCA GTT GGG AGA TTG GTC CTG TTA AGA TCT CCC CAG
              819         828         837         846         855         864
    AAT GGT CCT GTT TCC TGT CCT CAT CAT TCC CCT AAC CCA TCT TTG TGG GGT CCC
              873         882         891         900         909         918
    TTA AGA CTT TGG AGG ATG ACA GTC AGA CAG GAA GAG AAT ACT GAT CCT GGC ATA
              927         936         945         954         963         972
    TGT CTA AAT AAA TTC CCT AAA GCC ACA CCA CTG CCC AGA TAT GCC CAG CCA GTG
              981         990         999        1008        1017        1026
    TAA TCA GGG TGG GTG CCA ACA TGG CCT GGT GCC CAG GTT TCC ATC AGC TTA GGG
```

Fig. 39 (cont.)

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      1035        1044        1053        1062        1071        1080
GCT CCC GTG TCC CAT ACG CTG CTC TGA CTC TTT CCT TTC CAG CCC CTG ACA TCA
                                                    Thr Pro Asp Ile Asn

      1089        1098        1107        1116        1125        1134
ATC CTG CCT GGT ACA CGG GTC GTG GGA TCA GGC CTG TGG GCC GCT TCG GGA GGA
  Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly Arg Arg

      1143        1152        1161        1170        1179        1188
GGA GGG CAG CCC TGA GGG ATG TCA CCG GAC CTG GCC TGC GGT GCC GGC TAA GCT
  Arg Ala Ala Leu Arg Asp Val Thr Gly Pro Gly Leu Arg Cys Arg Leu Ser Cys

      1197        1206        1215        1224        1233        1242
GCT TCC CAC TGG ATG GAA GTG CCA AGT TCT CTC ACA GCT CAT GAA GAC AGT GAT
  Phe Pro Leu Asp Gly Ser Ala Lys Phe Ser His Ser Ser ***

      1251        1260        1269        1278        1287        1296
GCT GAG CCC AAG CCC ACC CTC CCT GTC CCC CCG CAG AGC CTC CTC CAC CCT CCC
      1305        1314        1323        1332        1341        1350
TTT CCG GTT TTC CCT CTG ATC TAA TAA AAG TGC TGG CTT TGT TTA TTG TAC ACT
      1359        1368        1377        1386        1395        1404
TGT AAC TAT GTG GTA ACA AAC CGG AAG GTG CTT TCT CTC TGG GGA GGG TAA CCA
      1413        1422        1431        1440        1449        1458
TGA AAG AAG CTC AGA ACC CAG TAA CCT CTT TGG AAA GAA GAA GCT CCC ACC TGC
      1467        1476        1485        1494        1503        1512
CCC CAA TAG AAC AAC TGA GAT CGC TCA TTA CCA GGC CCC ACA GAA GTT GTC CTG
      1521        1530        1539        1548        1557        1566
GTC CCT TAA GAC CCT GCA GTG GGG GAA GGG AAT GTT GAT TCA GTG TTC CTA TAA
      1574
ATT CCT GT 3'
```

Fig. 40

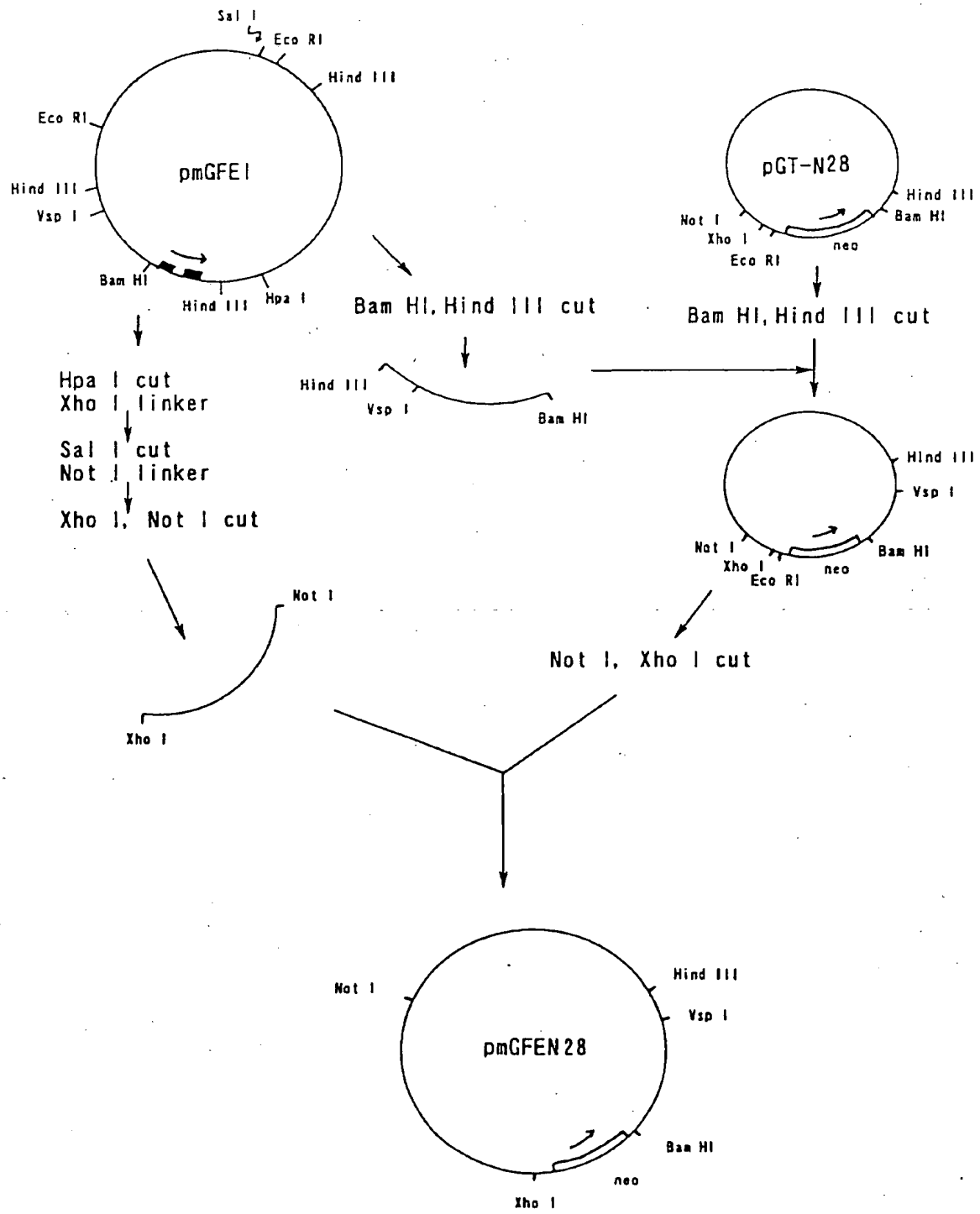
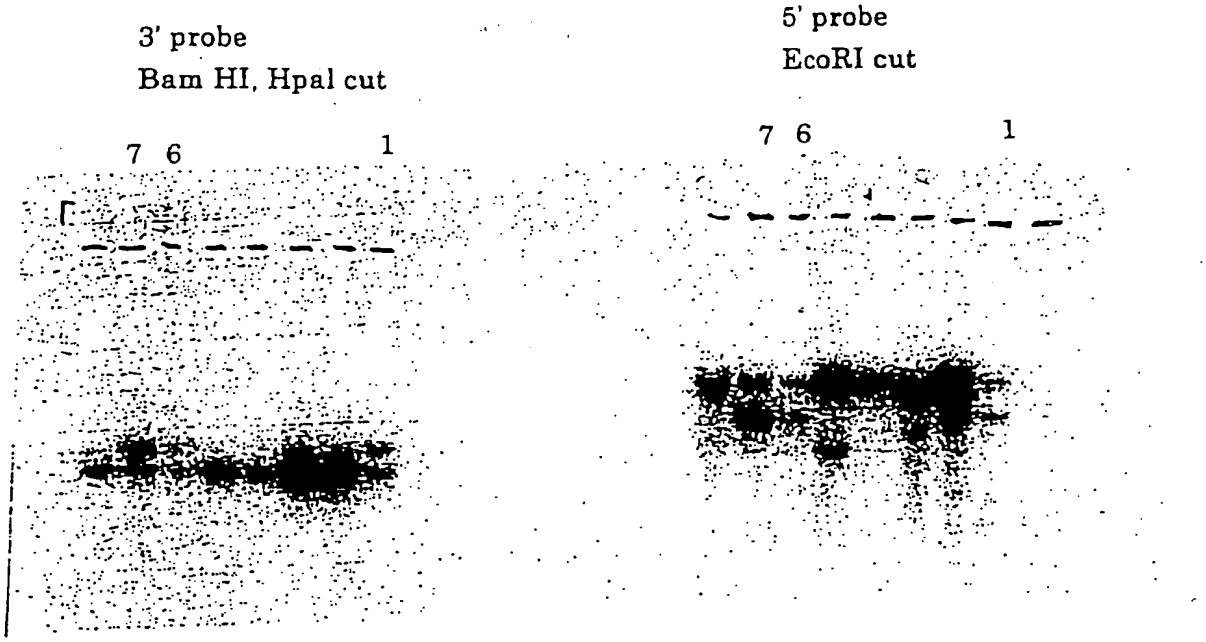
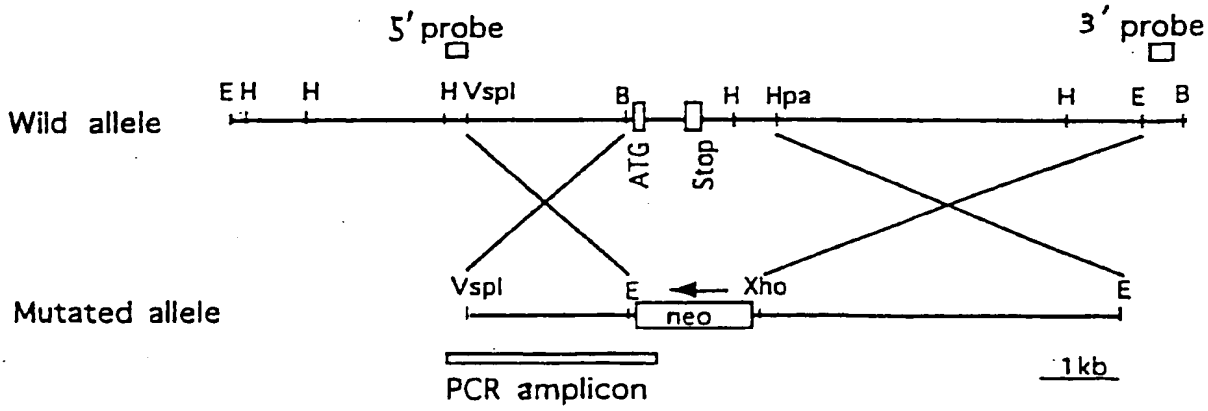


Fig. 41





INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/JP 98/01923

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K14/72 C12N1/21 C12N5/10
C07K16/18 C12Q1/68 G01N33/52 A61K38/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 08317 A (CHIRON CORP ;DUHL DAVID (US)) 6 March 1997 page 1, line 17; page 2,4; page 14, line 26; page 25, line 5-10; page 29,32-33; claims ---	1-5,10, 11
Y	WELCH S K ET AL: "SEQUENCE AND TISSUE DISTRIBUTION OF A CANDIDATE G-COUPLED RECEPTOR CLONED FROM RAT HYPOTHALAMUS" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 209, no. 2, 17 April 1995, pages 606-613, XP002032820 cited in the application see the whole document --- -/--	1-5,10, 11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 September 1998	25/09/1998

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	MARCHESE, A., ET AL. : "cloning and chromosomal mapping of three novel genes , GRP9, GRP10, and GRP14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors" GENOMICS, vol. 29, 1995, pages 335-344, XP002077455 cited in the application page 337-338; page 344; Fig. 1, 3	1-5,10, 11
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P,X	WO 97 24436 A (TAKEDA CHEMICAL INDUSTRIES LTD ;HINUMA SHUJI (JP); HABATA YUGO (JP)) 10 July 1997 see the whole document	1-5, 10-12
T	HINUMA, S., ET AL. : "a prolactin-releasing peptide in the brain" NATURE , vol. 393, 21 May 1998, pages 272-275, XP002077456 see the whole document	1-12

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Information on patent family members

Internal Application No
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